Infectious immunity and pneumococcal vaccine responses in multiple myeloma and related disorders

Johanna Karlsson

Department of Internal Medicine and Clinical Nutrition

Institute of Medicine

Sahlgrenska Academy at University of Gothenburg, Göteborg, Sweden

UNIVERSITY OF GOTHENBURG

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johanna.karlsson@vgregion.se

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To Hannes and Jakob

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Department of Internal Medicine and Clinical Nutrition, Institute of Medicine
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ABSTRACT

Multiple myeloma (MM), Waldenstrom's macroglobulinemia (WM), and monoclonal gammopathy of undetermined significance (MGUS) are B cell conditions associated with suppressed immune functions and susceptibility to infection. Severe pneumococcal disease is common not least in MM patients and vaccination is considered important, although the protective efficacy is debated. The aims of the first two studies of this thesis were to investigate humoral immunity to a spectrum of prevalent pathogens, and responses to pneumococcal vaccination with either a 23-valent polysaccharide vaccine or a 7-valent conjugated vaccine in elderly patients with MM, WM, and MGUS. We further compared two methods for evaluation of pneumococcal vaccine responses, serotype-specific ELISA and opsonophagocytosis (OPA) in the same groups of patients, and retrospectively examined the prevalence of respiratory viruses in MM. Background antibody levels to pathogens were most depressed in MM but low antibody levels were also seen in WM and MGUS compared to age-matched controls. Pneumococci, *Staphylococcus aureus*, varicella zoster virus, and fungi (Candida, Aspergillus) were identified as risk pathogens, while immunity to *Haemophilus influenzae* and most viruses was retained in all study groups. Likewise, responses to pneumococcal vaccination were suppressed in all three patient categories. No differences between the vaccine types given as single doses were found. Pneumococcal antibody titers as measured by ELISA and OPA correlated very poorly in MM and WM patients, and our data indicate that ELISA measurements may overestimate anti-pneumococcal immunity in these patients. Rhinovirus, influenza virus and respiratory syncytial virus were the most commonly detected respiratory viruses in the investigated MM cohort. Patients with virus-positive tests were younger and had shorter disease duration than patients with negative analyses. In summary, patients with MM, WM and MGUS have a suppressed humoral immunity to many common pathogens, foremost bacteria. Reduced responses to pneumococcal vaccination can be expected in these patients. The use of an OPA method should be preferred for evaluating pneumococcal vaccine responses in B cell malignancies.

Keywords: Multiple myeloma, Waldenstrom's macroglobulinemia, MGUS, elderly, immunity, infection, pneumococcal vaccination, ELISA, opsonophagocytosis
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Sammanfattning

Multipelt myelom, Waldenströms makroglobulinemi (WM) och monoklonal gammopati av oklar signifikans (MGUS) är tillstånd som drabbar immunförsvarets B-celler. Medan multipelt myelom och WM är blodcancerformer är MGUS ett icke malignt (elakartat) tillstånd, som dock kan vidareutvecklas till en malign sjukdom, i första hand myelom eller WM. Dessa tillstånd drabbar huvudsakligen äldre personer, och medelåldern för insjuknande i myelom och WM är 70 år. Multipelt myelom är en av de vanligaste blodcancerformerna medan WM endast drabbar ca 4 personer per miljon invånare. MGUS kan påvisas hos 3% av befolkningen över 50 år med en ökande incidens vid ökande ålder. Tillstånden karakteriseras av en tillväxande klon av B-celler som alla producerar likadana antikroppar, oftast icke-funktionella, den så kallade M-komponenten. Denna klon av B-celler tränger undan friska B-celler, vilket får till följd att en minskad mängd funktionella antikroppar produceras. Detta parallellt med tillkomst av andra defekter i immunförsvaret gör patienterna infektionskänsliga, och infektioner är en av de vanligaste dödsorsakerna vid multipelt myelom. En ökad sjuklighet och dödlighet i infektioner har även visats för WM och MGUS.

Kapselförsedda bakterier såsom pneumokocker är viktiga sjukdomsalstrare vid B-cellssjukdomar. Pneumokocker orsakar i första hand lunginflammation men så kallade invasiva infektioner, blodförgiftning och hjärnhinneinflammation, är inte ovanliga och dödligheten i dessa är hög i synnerhet hos äldre personer. Vaccination mot pneumokocker ingår sedan 2009 i det svenska barnvaccinationsprogrammet och har lett till betydande minskning av svåra pneumokockinfektioner i samhället. Pneumokock-vaccination rekommenderas också till riskgrupper, såsom äldre och personer med försämrat immunförsvar (där B-cellssjukdomar ingår). Dock har ett nedsatt vaccinationssvar tidigare setts inte minst hos personer med multipelt myelom.

I denna avhandling studeras infektionskänslighet samt vaccinationssvar mot pneumokocker hos äldre (> 60 år) västsvenska patienter med ovan nämnda diagnoser. I det första delarbetet har vi mätt bakgrundsnivåer av antikroppar i blodet mot ett stort antal bakterier, virus och svampar hos patienterna och jämfört med en åldersmatchad kontrollgrupp utan blodsjukdom. Syftet var att undersöka om och hur immunförsvaret är nedsatt i de tre sjukdomsgrupperna och att identifiera smittämnen som dessa patienter har ett tydligt nedsatt skydd emot. I delarbete II undersöks svaret på pneumokockvaccination hos samma patienter. Vaccin mot pneumokocker finns av två principiellt skilda typer, dels polysackaridvaccin, vilket varit standardvaccinet till vuxna, dels konjugatvaccin, som utvecklats för att ge ett gott vaccinationsskydd hos små barn. Det sistnämnda har i vissa studier visat en bättre effekt även hos äldre och personer med nedsättning av immunförsvaret. Vi ville därför jämföra de två vaccinerna i våra patientgrupper. I det tredje delarbetet jämförs två metoder för utvärdering av vaccinsvar

mot pneumokocker, antikroppsmätning med standardmetoden ELISA och en funktionell analys, opsonofagocytos (OPA), där man mäter hur väl patientens blod avdödar bakterierna. Det fjärde delarbetet ägnas enbart åt patienter med multipelt myelom, hos vilka vi retrospektivt studerat förekomsten av luftvägsvirus i samband med luftvägssymtom. Vi ville också undersöka om man kunde finna en koppling mellan positivt virustest och andra faktorer som sjukhusvård, dödlighet, ålder och sjukdomsaktivitet.

Påtagligt sänkta antikroppsnivåer mot ett flertal smittämnen påvisades hos patienter med multipelt myelom, men även de övriga två patientgrupperna hade lägre nivåer än kontrollgruppen. Detta var mest påtagligt för pneumokocker och gula stafylokocker (hudbakterier) liksom för vattkoppsvirus och svampar. Tvärtom förelåg bibehållna antikroppsnivåer mot flertalet virus i alla patientgrupper. I samtliga patientgrupper sågs också ett nedsatt vaccinationssvar mot pneumokocker, mest uttalat hos myelom-patienterna. Vi kunde inte påvisa någon skillnad i svaret på de två olika vaccintyperna givna som enkeldos för någon av studiegrupperna inklusive kontrollgruppen. Överensstämmelsen mellan de två metoderna för mätning av vaccinationssvar mot pneumokocker var god i kontrollgruppen och hos patienter med MGUS men mycket dålig hos patienter med myelom och WM. Våra data inger misstanke om falskt höga antikroppsnivåer mätt med ELISA hos vissa av dessa patienter, och vi rekommenderar därför inte ELISA vid dessa diagnoser. De oftast påvisade luftvägsvirusen i den undersökta kohorten av myelompatienter var rhinovirus, influensavirus och respiratoriskt syncytievirus (RS). Patienter med positivt virustest var yngre och hade kortare sjukdomsduration än patienter med negativ analys.

Sammanfattningsvis visar dessa studier ett nedsatt antikroppsskydd mot ett flertal vanliga smittämnen, framför allt bakterier, samt ett nedsatt vaccinationssvar mot pneumokocker hos patienter med multipelt myelom, Waldenströms makroglobulinemi och MGUS. Vid mätning av pneumokockvaccinationssvar hos patienter med myelom och WM är en funktionell OPA-analys att föredra framför ELISA.

List of publications

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Comparative study of immune status to infectious agents in elderly patients with multiple myeloma, Waldenstrom's macroglobulinemia, and monoclonal gammopathy of undetermined significance.**
Karlsson J, Andréasson B, Kondori N, Erman E, Riesbeck K, Hogevik H, Wennerås C.
Clin Vaccine Immunol 2011;18(6):969-977.

II. **Pneumococcal vaccine responses in elderly patients with multiple myeloma, Waldenstrom's macroglobulinemia, and monoclonal gammopathy of undetermined significance.**
Karlsson J, Hogevik H, Andersson K, Roshani L, Andréasson B, Wennerås C.
Trials Vaccinol 2013;2:31-38.

III. **Poor correlation between pneumococcal IgG and IgM titers and opsonophagocytic activity in vaccinated patients with multiple myeloma and Waldenstrom's macroglobulinemia.**
Karlsson J, Roalfe L, Hogevik H, Zancolli M, Andréasson B, Goldblatt D, Wennerås C.
Clin Vaccine Immunol 2016; 23(4):379-385.

IV. **Respiratory viruses in multiple myeloma: A single-center epidemiological study.**
Karlsson J, Blimark C, Hogevik H, Wennerås C, Andréasson B.
In manuscript.

Content

Abbreviations

ASCT	Autologous stem cell transplantation
CAPiTA	Community-acquired Pneumonia Immunization Trial in Adults
CD	Cluster of differentiation
CD40L	CD40 ligand
CDC	Centers for Disease Control and Prevention
CMV	Cytomegalovirus
CoV	Coronavirus
CPS	C-polysaccharide
CRP	C-reactive protein
Ct	Cycle threshold
DAMPs	Danger-associated molecular patterns
DC	Dendritic cell
EBV	Epstein Barr virus
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment antigen binding
Fc	Fragment crystallizable
FLC	Free light chains
HBSS	Hanks' balanced salt solution
HHV	Human herpes virus
Hib	*Haemophilus influenzae* type b
HIV	Human immunodeficiency virus
HL	Human leukocyte
HR	Hazard ratio
HSV	Herpes simples virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMiDs	Immunomodulatory drugs
IPD	Invasive pneumococcal disease
IVIg	Intravenous immunoglobulin
LytA	Autolysin
MAPK	Mitogen-activated protein kinase
MASP	MBL-associated serine protease
MBL	Mannose-binding lectin
MGUS	Monoclonal gammopathy of undetermined significance
MHC	Major histocompatibility complex
MM	Multiple myeloma
M-protein	Monoclonal protein
MyD	Myeloid differentiation
NF	Nuclear factor
NK cell	Natural killer cell
OD	Optical density
OPA	Opsonophagocytosis assay
O-PLS	Orthogonal Partial Least Squares

PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PET-CT	Positron emission tomography-computed tomography
PCV	Pneumococcal conjugate vaccine
PPV	Pneumococcal polysaccharide vaccine
PRR	Pattern recognition receptor
Psp	Pneumococcal surface protein
RNA	Ribonucleic acid
RT	Real time
RV	Respiratory virus
Th cell	T helper cell
THYE	Todd-Hewitt yeast broth extract
TLR	Toll-like receptor
TMP-SMX	Trimethoprim-sulfamethoxazole
TNF	Tumor necrosis factor
Treg	T regulatory cell
VZV	Varicella zoster virus
WBC	White blood cell counts
WHO	World Health Organization
WM	Waldenstrom's macroglobulinemia

1 Introduction

Multiple myeloma, Waldenstrom's macroglobulinemia, and monoclonal gammopathy of undetermined significance (MGUS) are conditions that belong to the monoclonal gammopathies. This group of disorders is defined by the expansion in the bone marrow of a single clone of B cells, which in its turn produces the disease-defining homogenous M- (monoclonal) protein that can be detected in the blood.[1, 2] The M-protein consists of identical antibodies of the IgG, IgA, IgM, IgD, or IgE isotype, and the size of the clone is a marker of the disease burden. Multiple myeloma and Waldenstrom's macroglobulinemia are malignant conditions. In contrast, MGUS is characterized by the presence of a low level of M-protein but no clinical signs of malignant disease; it may, however, progress into a malignant state, most commonly multiple myeloma or Waldenstrom's macroglobulinemia.[1] Conversely, virtually all cases of multiple myeloma are preceded by MGUS.[3]

Patients with B cell malignancies and disorders are immune deficient and have an increased susceptibility to and mortality in infections.[4-6] Their immunosuppression derives from the displacement of non-malignant B cells and concomitantly reduced levels of functional antibodies, abnormalities of other cell lines of the acquired and innate immune systems, as well as treatment-induced suppression of immune functions.

Several studies have shown an increased incidence of infections such as pneumonia, sepsis, influenza, and herpes zoster as well as a diversity of autoimmune and inflammatory disorders in patients who have subsequently been diagnosed with multiple myeloma, Waldenstrom's macroglobulinemia, or MGUS.[7-10] This could indicate an underlying immune disturbance present several years before the hematological diagnosis but also raises the possibility that immune-related conditions may act as triggers for the development of a B cell malignancy or disorder.

1.1 Multiple myeloma

Multiple myeloma is a disease of long history. Bone lesions suggestive of the diagnosis have been identified in skeletons of several thousand years of age in Egypt and European countries.[11] The first documented cases of multiple myeloma were described in the middle of the 19th century in patients suffering from fatigue, back pain, repeated fractures, and peripheral edema. The myeloma-specific proteinuria was described by Henry Bence Jones in 1848. Half a decade later, the characteristics of plasma cells were known and this type of cell was defined as the proliferating cell line in myeloma.[11] The Swedish hematologist Jan Waldenström first described the M-protein typical of multiple myeloma and other monoclonal gammopathies, seen as a narrow band on serum-electrophoresis.[12]

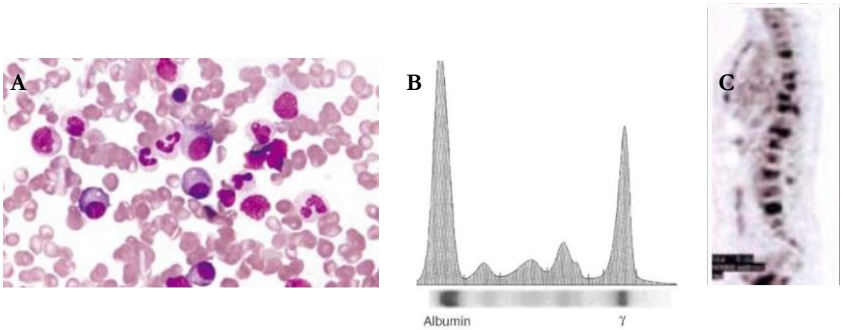

Figure 1. *(A) Plasmacytosis in bone marrow. (B) Serum-electrophoresis showing a narrow band in the γ region indicative of the presence of a monoclonal (M-) protein. (C) Vertebral osteolytic bone lesions (black) on PET-CT. Figures (A) and (C) are reproduced and adapted with permission of the WJG Press and Baishideng.[13] Figure (B) is used with permission from Wiley and sons.[1]*

Multiple myeloma is the second most common hematological malignancy after lymphoma. It accounts for 10-15% of hematological cancer and 1% of all types of malignancy,[14] and has an incidence of about 6 cases per 100.000 inhabitants in Sweden.[15] The incidence increases with age, and patients under the age of 40 are very rare. The median age at diagnosis is about 70 years. Multiple myeloma is somewhat more common in males than in females.[14, 16]

1.1.1 Definition

A diagnosis of multiple myeloma requires the presence of at least 10% of clonal bone marrow plasma cells or extramedullary clonal plasma cells in a biopsy (plasmacytoma) and one or more myeloma-defining events. These events represent end-organ damage that can be attributed to the underlying plasma cell disorder and include hypercalcemia, renal insufficiency, anemia, and osteolytic bone lesions on skeletal radiography.[17] Although most patients with multiple myeloma present with symptoms, the disease can in an early stage be silent, so called smouldering myeloma. This state is defined by serum

M-protein > 30 g/L and/or clonal bone marrow plasma cells ≥ 10% but absence of myeloma-defining events.[17] The M-protein secreted in multiple myeloma is commonly of the IgG, IgA or IgD isotype, or consists only of immunoglobulin free light chains (κ and λ). Non-secretory myeloma are also seen.[14]

1.1.2 Clinical features

Skeletal pain is the most common symptom of multiple myeloma. It is a result of plasma cell infiltration of the bone marrow causing osteolytic lesions and pathological fractures.[15] The bone resorption often results in hypercalcemia which, together with toxic effects of immunoglobulin light chains, causes renal failure. This is seen in about 25% of patients at diagnosis and might require dialysis.[4] Fatigue is another common symptom usually related to anemia, which is found in about 70% of patients at diagnosis and is caused by the bone marrow infiltration of plasma cells. This might also lead to neutropenia and thrombocytopenia.[14] Recurrent and severe infections are seen in many patients especially during progressive disease.[4, 18, 19]

1.1.3 Treatment and prognosis

There is no curative treatment for multiple myeloma but treatment options have greatly improved during the last 20 years and resulted in prolonged periods of remission and survival.[20] Despite this, overall survival in multiple myeloma is only 4-5 years.[21] Renal failure, male sex, and high age are associated with a poorer prognosis.[4, 16] The indication for treatment is symptomatic disease (or high-risk smouldering myeloma as defined by biomarkers). High-dose melphalan with autologous stem cell transplantation (ASCT) is the established treatment for patients younger than 65-70 years since the end of the 1990s. This was the first break-through in the treatment of multiple myeloma since the 1960s and has been followed by the introduction of immunomodulatory drugs (IMiDs) such as thalidomide and lenalidomide and the proteasome inhibitors bortezomib and carfilzomib. For elderly patients, various combinations of chemotherapy and corticosteroids, often melphalan and prednisone (MP), and any of the new drugs are used. At relapse, ASCT can be repeated if the patient responded well the first time with a long so called plateau phase without signs of organ damage or symptoms. Otherwise various combinations of chemotherapy and new drugs are used.[15]

1.2 Waldenstrom's macroglobulinemia

Waldenstrom's macroglobulinemia (WM) is named after Jan Waldenström, who made the original description of the disease in 1944 and reported two patients with oronasal bleeding, lymphadenopathy, anemia, thrombocytopenia, hyperviscosity, and an elevated erythrocyte sedimentation rate.[22] The condition is classified as a lymphoplasmacytic lymphoma with an IgM monoclonal gammopathy.[23] WM is a rare disease with an incidence of about 4 cases per million inhabitants.[24] It constitutes 1-2% of all

hematological malignancies.[25] The median age at diagnosis is around 70 years, and the incidence is twice as high in men as in women. Familial cases of WM have been reported and have been associated with a high incidence of autoimmune disorders.[26, 27] Also, an increased risk of WM has been described for patients with various infections in their personal history suggesting that chronic immune stimulation may be associated with the development of the disease besides genetic factors.[9]

Figure 2. *Jan Waldenström 1963. Reprinted with permission of Elsevier.*[28]

1.2.1 Definition

WM is a lymphoproliferative tumor defined by the infiltration of clonal lympho-plasmacytic cells (small B lymphocytes) in the bone marrow and sometimes in other lymphatic tissues such as lymph nodes and the spleen, with the presence of a monoclonal immunoglobulin (M-protein) of IgM-type in the blood.[2, 23] Since these criteria do not define a specific level of M-protein and tumor cell infiltration of the bone marrow, and many patients are asymptomatic at diagnosis, the distinction between IgM MGUS and WM is not evident but more of a continuum.[29]

1.2.2 Clinical features

Presenting symptoms are in many cases related to tumor infiltration, and fatigue, weight loss and anemia are common.[25] Thrombocytopenia causes nose bleeds and petecchiae. Hepato-splenomegaly and lymphadenopathy are common. Hyperviscosity is a central feature and is caused by the IgM M-protein, a large molecule that makes the blood more viscous. This may give rise to symptoms including headache, blurring of vision, vertigo, severe bleedings, thrombosis, and confusion.[29] Peripheral neuropathy is seen in about 20% of the patients as an autoimmune effect of the M-protein; other autoimmune symptoms include vasculitis, cryoglobulinemia, and hemolytic anemia.

1.2.3 Treatment and prognosis

WM is a chronic disease and no curative treatment is available. However, overall survival has improved significantly during the last 30 years probably due to new treatment strategies.[30] Negative prognostic factors are high age at diagnosis, anemia, thrombocytopenia, high levels of M-protein, and signs of renal failure. In patients with two risk factors the overall 5-year survival is still almost 70%.[29] Only symptomatic patients should be treated. If there are signs of hyperviscosity, plasma exchange should always be considered. The standard primary treatment includes the anti-CD20 monoclonal rituximab, most often in combination with cyclophosphamide and high-dose steroids. Novel agents such as bendamustin and bortezomib can be added.[29, 31] ASCT is recommended for younger patients who do not respond to the first line treatment or relapse quickly.[29]

1.3 Monoclonal gammopathy of undetermined significance (MGUS)

The prevalence of MGUS in the population above 50 years of age is around 3%, and rises with increasing age. The condition is more common in males than in females.[32] There is evidence of a familial predisposition for MGUS and other lymphoproliferative or plasma cell disorders.[33] An increased incidence has also been described among certain groups of immunocompromised patients, for example HIV-positive individuals and renal transplant recipients, and as a consequence of environmental factors such as occupational exposure to pesticides and petroleum products.[32]

Patients with MGUS have a 25-fold increased risk of developing multiple myeloma, and a 46-fold increased risk of WM.[34] Due to the potential of malignant transformation, MGUS patients need life-long follow-up with regular controls of their serum M-protein levels and clinical examinations.

1.3.1 Definition

A diagnosis of MGUS requires a serum M-protein concentration of less than 30 g/L, < 10% clonal plasma cells in the bone marrow, and no end-organ damage (*i.e.*, no hypercalcemia, renal insufficiency, anemia, or osteolytic bone lesions).[17] Around 15% of MGUS cases have a lymphoplasmacytic cell clone in the bone marrow and secrete M-protein of IgM-type; the remaining 85% are of clonal plasma cell origin and secrete IgG or IgA M-protein.[35]

1.3.2 Clinical features

MGUS is commonly diagnosed during a medical examination for another cause, typically the finding of an elevated erythrocyte sedimentation rate or an abnormal serum

electrophoresis. Although MGUS is per definition an asymptomatic disorder there is evidence of increased co-morbidity regarding conditions such as skeletal disease (osteoporosis and fractures of hip and vertebrae), polyneuropathy, thromboembolism, dermatologic conditions, and bacterial as well as viral infections.[1, 6, 36, 37]

1.3.3 Prognosis

Patients with MGUS have an average annual risk of progression to multiple myeloma, Waldenstrom's macroglobulinemia and other lymphoproliferative diseases of 1%. This risk remains even after 25 years of a stable gammopathy. However, the probability of progression is highly heterogeneous, and the majority of MGUS patients will never develop a lymphoproliferative malignancy.[34] Rajkumar *et al.* defined three risk criteria for malignant transformation; a serum M-protein of ≥ 15 g/L, IgA or IgM MGUS, and an abnormal serum free light chain (FLC; κ and λ) ratio. Patients with all three risk factors had a risk of progression of 58% at 20 years from diagnosis while only 5% of patients without risk factors progressed during the same time period.[38]

Kristinsson *et al.* found a poorer survival among MGUS patients than in the general population, specifically among elderly patients.[39] The excess mortality in MGUS was not only due to progression to hematological malignancy. Patients with a diagnosis of MGUS also had a higher risk of dying from bacterial infections (hazard ratio [HR] = 3.4), myeloid malignancies, heart disease, and liver and kidney diseases. Younger patients more often died from lymphoproliferative diseases while cardiovascular diseases dominated among the elderly.[39]

1.4 Infectious defense

The human defense against infection can be divided into three arms: Functional barriers, innate immunity, and acquired or adaptive immunity. Barriers protecting against infection can be chemical, such as the hydrochloric acid in the stomach and lysozyme in the saliva, and mechanical such as the mucus layer covering the respiratory and gut epithelium. The innate and acquired immune systems consist of cells and molecules that recognize and react upon danger signals in the form of foreign pathogens or injured tissue. The cells involved are mainly white blood cells (neutrophilic and eosinophilic granulocytes, monocytes, basophilic granulocytes, mast cells, B- and T lymphocytes, and natural killer cells) derived from multipotential hematopoietic stem cells in the bone marrow. The hematopoiesis is summarized in Figure 3.

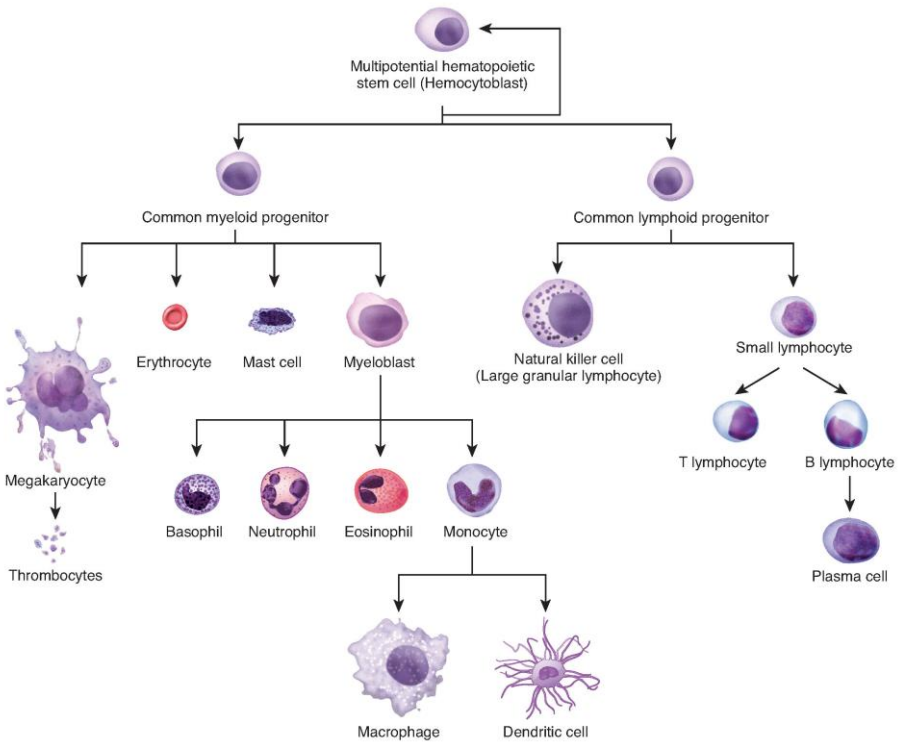

Figure 3. *The normal hematopoiesis in adults. Reprinted with permission of OpenStax.*[40] *The figure may be downloaded for free at http://cnx.org/contents/14fb4ad7-39a1-4eee-ab6e-3ef2482e3e22@6.27.*

1.4.1 Innate immunity

The innate immune system is present in the host at birth and offers an immediate response to foreign antigens through an inflammatory process.

This system recognizes a limited number of highly conserved structures that are common to different microbes, for example lipopolysaccharide and peptidoglycan present on bacteria, and viral RNA, but also endogenous danger elements in the form of injured or dead tissue. The microbial molecules are referred to as pathogen-associated molecular patterns (PAMPs), endogenous trigger molecules as danger-associated molecular patterns (DAMPs).[41, 42] The system is activated through pattern recognition receptors (PRRs).[41] These may be secreted, for example C-reactive protein (CRP) and mannose-binding lectin (MBL), which bind to bacteria and may activate the complement system.[43] Other PRRs are bound to the surface of immune cells or are intracellular. The most well-known are the toll-like receptors (TLRs) which include surface as well as intracellular receptors that bind to bacterial components and viral RNA. The receptors signal via adaptor molecules, for example MyD88 which activates MAP kinases and the transcription factor NFκB that induce the production of cytokines such as interleukin-1 (IL-1), tumor necrosis factor (TNF), and IL-6. Soluble mediators including cytokines, prostaglandins, and oxygen radicals that are released as part of the inflammatory process play a key role in the recruiting of other immune cells as well as triggering of the adaptive immune system.

Cells involved in innate immunity include the neutrophils, eosinophils and basophils, monocytes, macrophages, dendritic cells, mast cells, and the natural killer (NK) cells.[44] Opposite to the adaptive immune system, innate immunity has no immunologic memory.

PHAGOCYTOSIS AND OPSONIZATION

There are several mechanisms by which the innate immune system rids the body of invaders, including phagocytosis, complement activation, and inhibition of virus replication.

Phagocytosis is, together with the complement system, the most important anti-bacterial systems in the human. It is exerted predominantly by macrophages and neutrophilic granulocytes. After adhesion to the phagocyte cell surface, the foreign body is engulfed in a vesicle, a phagosome, in the cytoplasm of the cell to which toxic substances such as lysozyme and defensins are recruited and eventually kill the intruder. The production of toxic oxygen radicals is crucial in the killing process.[44]

Macrophages can be activated by direct contact with the microbe, but also by the binding of a microbe to a PRR. Opsonization is a way of facilitating phagocytosis, particularly of encapsulated organisms. The foreign body is covered by opsonins, either

antibodies of the IgG isotype or by C3b complement fragments, which in their turn bind to specific receptors (Fcγ, C3b receptors) on the phagocytosing cell (macrophages, neutrophils). Opsonization also enables phagocytes to recognize pathogens that do not express PAMPs, for example viruses.[45] Pneumococci are difficult targets due to the polysaccharide capsule that surrounds the bacteria and protects against phagocytosis.[46] In this case, opsonization with complement factors (the classical pathway; see below) and antibodies is required for effective killing. Conversely, patients with antibody or complement deficiencies are at increased risk of developing severe pneumococcal infections.[47]

Dendritic cells also have phagocytic capacity. However, their main function is antigen-presentation whereby they act as a bridge to the adaptive immune system. NK cells kill their targets, typically virus-infected or malignant cells, through induction of apoptosis or directly by secretion of cytotoxic substances such as perforins and granzymes without previous antigen presentation.[48]

COMPLEMENT

The complement system is a series of plasma proteins that plays an essential role in innate immunity by facilitating phagocytosis, attracting leukocytes, and directly lysing microbes. Many of the complement factors are proteases, which cleave and activate the next member of the system. The central process is the cleavage of factor C3 into C3a and C3b, after which the C3b fragment can opsonize foreign bodies, thereby dramatically increasing phagocytosis.

There are three different pathways by which the complement system is activated: the classical pathway, the alternate pathway, and the lectin-binding pathway. The lectin-binding pathway uses the complement factor mannose-binding lectin (MBL) that binds to carbohydrate surface structures of bacteria and fungi. This results in the activation of an enzyme, MBL-associated serine protease (MASP), which cleaves factors C4 and C2. An enzyme complex, C4b2a, is created, which in turn cleaves factor C3.[45]

The classical pathway is activated by antibodies bound to an antigen, for example bacteria. Antibodies of the IgG and IgM isotypes have binding sites for the complement factor C1q. When at least two arms of immunoglobulins are cross-linked by C1q molecules, the C1 complex containing C1q, C1r and C1s is activated. C1s cleaves factors C4 and C2, and as in the lectin-binding pathway, the C4b2a enzyme complex cleaves factor C3.[45] Since the IgM molecule has many binding sites for complement it is a much more potent activator of the complement system than IgG. The classical pathway is essential for the phagocytosis of encapsulated bacteria such as pneumococci.

The alternate pathway is a reinforcing system of the other two pathways, and becomes activated as soon as C3b has bound to a surface. It may then bind factor B, which is

further cleaved by factor D. The remaining complex, C3bBb, converts C3 into C3a and C3b. The C3bBb convertase is very short-lived but is stabilized by binding to properdin, which prolongs the convertase's half-life 5- to 10-fold allowing for the production of larger amounts of the C3b opsonin.[45]

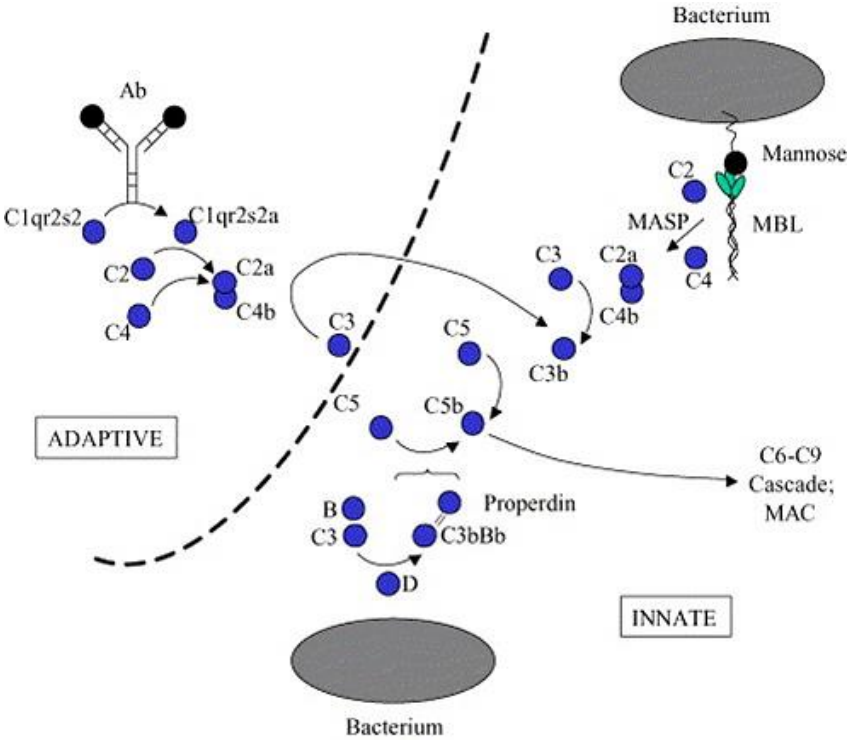

Figure 4. *The complement cascade. Reprinted with permission of Elsevier.*[44]

The direct cell lysing potential of the complement system is effectuated by the "membrane attack complex", which is composed of the terminal complement components C5-C9. Its formation is initiated by the cleavage of factor C5 by the binding of C3b and either of the classical or alternative pathways' C3 convertases. Factors C5-C8 create an anchor site for the C9 molecules, which form the actual attack complex that can penetrate and lyse gram-negative bacteria, and also inactivate viruses.[44]

The complement fragments C3a-C5a which are released during activation of the complement system are also called anaphylatoxins. C3a causes degranulation of mast cells with the release of histamine, which increases the permeability of blood vessels and allows for the spread of plasma proteins into the tissue.[45] C5a induces chemotaxis of neutrophils and monocytes, attracting them to areas of inflammation.

NATURAL IGM

IgM antibodies can be subdivided into natural and immune IgM. The latter are antigen-specific and produced upon exposure to specific pathogens (see below). In contrast, natural IgM antibodies are poly-reactive and can bind to a large number of conserved epitopes shared by diverse microorganisms. It acts as part of the first line defense against infections, and its receptor has been found on all lymphoid and myeloid cells but with the highest levels on B lymphocytes.[49] This low-affinity antibody type does not require antigen presentation and is already present at birth.

Natural IgM has been shown to protect against infection, to clear apoptotic and dying cells from the circulation, to regulate inflammation by binding to lymphocytes and inhibiting cell division or activation, but also to induce autoimmune disease.[49] Natural IgM has an important role in the early defense against pneumococci and other encapsulated bacteria. It has been shown to contribute to the protection against invasive disease by clearing bacteria from the circulation and delocalizing them to the marginal zone of the spleen.[50] Natural IgM is also capable of activating the classical complement pathway.[51]

1.4.2 Acquired immunity

In contrast to innate immunity, the acquired or adaptive immune system is not pre-formed at birth but educated upon encounter with antigens. It is also called specific immunity since the participating cells, the T and the B lymphocytes possess unique receptors on their cell membranes that recognize and distinguish individual antigenic epitopes, specific for each individual lymphocyte. The system has an almost unlimited capability of detecting new structures. The key feature of the acquired immune system is its memory function. A proportion of the lymphocytes that have reacted to an encountered antigen will develop into memory cells. These remain inactive until the next time the individual is exposed to the same antigen, when they provide a much faster and stronger response than at the primary encounter (a few hours vs. 1-2 weeks).

T CELLS

T cells recognize antigenic epitopes via a specific T cell receptor. Pre-T cells are formed in the bone marrow and migrate to the thymus, where T cell receptor rearrangement occurs and the cells develop into mature T lymphocytes; T cells reactive to self-antigens are sorted out.

Antigens are presented to the T lymphocytes by MHC (major histocompatibility complex) molecules. MHC class I can be found on most cell types in the human body while MHC class II molecules are foremost present on specific antigen-presenting cells that are part of the innate immune system, such as macrophages and dendritic cells. The T cells that attach to MHC class I have a CD8 co-receptor on their surface while those

that bind to MHC class II have a CD4 co-receptor. The CD4+ T cells secrete cytokines and chemokines that activate and recruit other immune cells such as macrophages, B lymphocytes, and cytotoxic (CD8+) T lymphocytes.[52] The CD8+ cells mainly act by secreting lytic proteins that destroy host cells infected by microbes.[53]

Upon antigenic stimulation, naïve CD4+ T cells differentiate into different subsets of T helper (Th) cells or regulatory T cells (Tregs), see Figure 5. Th1 T cells secrete interferon-γ (IFN-γ) that activates macrophages and dendritic cells and are important in the defense against intracellular pathogens such as *Mycobacterium tuberculosis*.[54] Th2 cells are maybe best known for their role in allergic diseases but are also active in the defense against parasite infections and in the activation of B lymphocytes, while theTh17 cells seem to have critical functions in the clearance of extracellular bacteria and fungi.[52] Tregs (CD4+CD25+) are crucial players in the suppression of auto-reactive T cells as well as in protecting the host against excessive immune responses towards foreign antigens.[55]

Naïve CD8+ T cells differentiate into cytotoxic T lymphocytes upon antigenic stimulation. Their lytic function is mediated by perforin release and the Fas-pathway, and is essential in the defense against viral infections since lysis of infected cells halts viral replication.[53]

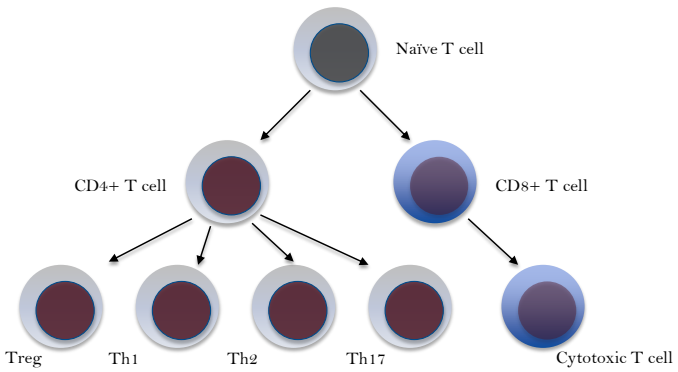

Figure 5. *T cell differentiation.*

B CELLS

The B cell recognizes its antigen by a membrane-bound antibody, the B cell receptor. Once a naïve B cell has encountered its specific antigenic epitope, it becomes activated and develops either into a plasma cell, which produces and secretes large amounts of the same type of antibody as its B cell receptor, or a memory B cell of the same specificity. The rearrangement of the B cell receptor occurs in the bone marrow. Activated B

(and T) lymphocytes primarily reside in the secondary lymphoid organs, the lymph glands and the spleen, which provide a milieu designed to facilitate the encounter with their cognate antigens. Eighty percent of the naïve B cells never locate their specific antigen and die about a week after leaving the bone marrow.[56]

The activation of B cells also requires the presence of T helper cells specific for the same antigen. In order to attract and activate these T cells the B lymphocytes can act as antigen-presenting cells by upregulating MHC class II molecules that present peptides of the antigen bound to the B cell receptor. The activated T helper cell secretes cytokines essential for B cell activation, typically IL-4, IL-6, and IL-10.[45] The process is enhanced by the expression of co-receptors on the B cell, for example CD40, which binds the CD40 ligand (CD40L) on the T helper cell. This binding promotes the switch of antibody isotypes from IgM to IgG, IgA, and IgE.[57]

Some antigens, such as the polysaccharide capsules of pneumococci and *Haemophilus influenzae* type b, can provide T cell-independent B cell activation. These antigens are typically large molecules with repetitive carbohydrate motifs that interact with multiple IgM B cell receptors simultaneously and induce a strong stimulating signal to the B cell. B cells expressing membrane receptors specific for such T cell-independent antigens reside in the marginal zone of the spleen and are able to provide a quick antibody response to blood-borne pathogens.[45]

ANTIBODIES

Antibodies are serum proteins that aid the host in the clearance of pathogens. They do not have a killing capacity of their own but exert their function by blocking, or neutralizing the microbe or toxin thereby preventing it to attach to its target cells, by facilitation of phagocytosis through opsonization, and by complement activation.[45]

The antibody consists of two identical heavy chains and two identical light chains. The chains have variable (V) and constant (C) domains. There are five variations of the constant domains of the heavy chains, which form the basis for the five immunoglobulin classes produced (IgM, IgG, IgA, IgE, IgD). Two classes of light chains exist, κ (kappa) and λ (lambda). The highly variable antigen-binding end of the antibody is called the Fab region, the constant "tail", which binds the effector cells, is named the Fc region.

The different immunoglobulin classes have different functions and sites where they are likely to reside. IgM is the largest molecule and consists of five antibody units joined by a J chain. It can bind at least five antigens simultaneously thus providing effective blocking and aggregation of pathogens. The Fc region of IgM and IgG has a binding site for complement factor C1; IgM is the most potent complement activator.[45]

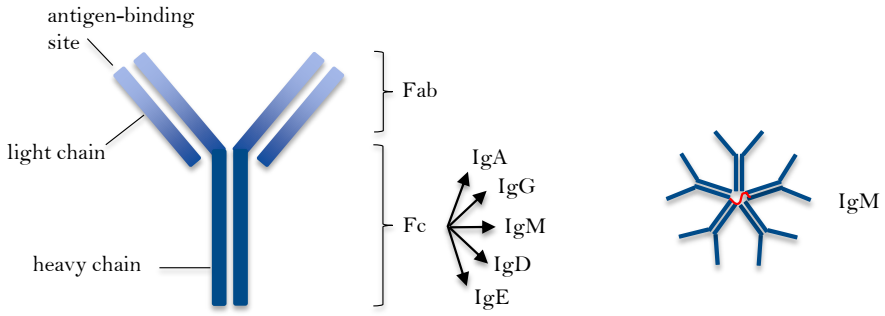

Figure 6. *Schematic design of an antibody and the formation of IgM. Fab, fragment antigen binding; Fc, fragment crystallizable.*

IgG is the dominating isotype in serum and the only antibody class that can cross the placenta.[58] Four subclasses of IgG, IgG1-4, exist in humans. IgG1 and IgG3 have high complement activating capacity, while IgG2 fixes complement poorly and IgG4 not at all. IgG antibodies to protein antigens are typically IgG1 and IgG3, while polysaccharides tend to give rise to an IgG2 response.[45, 59, 60] IgG2 subclass deficiency may therefore cause an increased susceptibility to encapsulated bacteria such as pneumococci.[61]

IgA exists in a monomeric form in serum and as secretory IgA on mucosal surfaces. Dimeric IgA is secreted by submucosal plasma cells and binds to "secretory component" on the basolateral side of epithelial cells. This complex is released into mucosal secretions, and the secretory component protects the antibody from cleavage by enzymes and gastric acid. IgA has poor capacity of inducing inflammation but effectively blocks the binding of bacteria, viruses, and toxins to target cells on mucosal surfaces.[45]

IgE binds to Fc receptors on mast cells and is responsible for allergic and anaphylactic reactions. IgD is expressed on the B cell membrane early during B cell differentiation and only small amounts are found in the serum; its function is largely unknown.

At the primary encounter with a pathogen, mainly IgM antibodies are produced. However, as the immune response progresses, some B cells switch to the other Ig classes (isotype switch). In parallel, the binding affinity of the antibody for the antigen increases by a process named somatic hypermutation. These processes occur in the germinal centers of lymph nodes and the spleen. Antibody affinity refers to the strength of the specific antibody-antigen binding. Antibody avidity is a wider concept influenced both by the antibody affinity and the contribution of multiple binding sites on the same antibody.

If a primary B cell response develops successfully, it is expected that upon renewed exposure to the same antigen, the secondary response will feature an IgM response of the same magnitude and duration, with a much more rapid and intense high-affinity IgG (or A, E) response.

1.4.3 B cell memory

Humoral immunity relies on B cell memory function, which is maintained by two different kinds of cells: the long-lived plasma cells and the memory B cells. The long-lived plasma cells reside in the bone marrow and may survive for months and years, in contrast to the short-lived plasma cells which have half-lives of only a few days.[62] The long-lived plasma cells have down-regulated their surface antigen receptors and can thus not be stimulated by antigen re-exposure. Instead they provide a constant release of high-affinity serum antibodies, which offers a first-line defense upon renewed encounter with known microbial antigens.[63]

Memory B cells are responsible for the rapid increase of specific antibodies at re-exposure to a previously encountered antigen by differentiation into short-lived plasma cells. The memory B cells are possibly also involved in replenishing the pool of long-lived plasma cells in the absence of pathogens.[64] Most memory B cells are isotype-switched and produce protective antibodies of mainly the IgG isotype. Another population, called IgM memory B cells, has been identified in the peripheral blood. These cells may be generated independently of germinal centers and have been shown to undergo somatic hypermutation but not isotype switch. They are involved in antibody production against T cell-independent antigens only, for example pneumococcal capsular polysaccharides.[65]

1.4.4 Pneumococcal immunity

Phagocytosis of bacteria opsonized by complement or antibodies is the classical host defense mechanism against pneumococci. Its importance is illustrated by the increased risk of pneumococcal infection seen in patients with complement or antibody deficiencies. Complement activation is primarily induced by the classical pathway and is enhanced by natural IgM.[47] C-reactive protein (CRP) is a soluble PRR of significance in the defense against pneumococci. Its name derives from the fact that it binds to pneumococcal cell wall constituent C-polysaccharide (CPS), whereupon complement is activated. Several TLRs are activated by pneumococci, for example TLR2 and TLR4, and deficiencies in their respective downstream intracellular signaling systems have been associated with an increased risk of pneumococcal infections.[47] B cells are crucial in the defense against pneumococcal infections. Specific anti-capsular antibodies are produced within a week after the onset of infection and induce an effective opsonophagocytic clearance of the bacteria.[45] They have also been shown to contribute to resistance against pneumococcal colonization.[66] Non-capsular antibodies directed towards various

components of the pneumococcal cell wall, such as CPS, are thought to contribute to this as well as to protection against invasive disease.[66, 67] In parallel, it is believed that CD4+ T cells play an important role in the acquired immunity against pneumococci, which may be illustrated by the highly increased risk of pneumococcal infection in untreated HIV patients with severe CD4+ T cell deficiency. Pneumococcus-specific CD4+ TH17 cells have been proposed as effector cells involved in reducing the duration of pneumococcal carriage and possibly also in preventing non-invasive pneumococcal disease.[67]

1.4.5 Vaccination

Vaccines based on plain polysaccharide antigens act in a T cell-independent manner, since T cells are programmed only to recognize linear protein fragments, peptides. Because of the lack of T cell secondary signals, B cells producing antibodies to polysaccharides cannot generate the classical immunologic memory emanating from IgG-switched memory B cells with high antibody affinity. The effect of polysaccharide vaccines is typically short-lived and cannot be boosted. Polysaccharides activate B cells of the splenic marginal zone.[68] These cells do not mature until the second year of life, and polysaccharide vaccines are thus poorly immunogenic in infants. The specific subset of IgM memory B cells has been identified as a potential circulating counterpart of marginal zone B cells,[69, 70] and patients with decreased or low levels of this B cell type, including splenectomized persons, infants < 2 years of age, and the elderly respond poorly to polysaccharide vaccines.[71]

To overcome the inherent polysaccharide vaccine resistance of infants, conjugated polysaccharide vaccines have been developed. These vaccines contain a protein carrier, for example tetanus or diphtheria toxoid, that is covalently linked to the polysaccharide antigen and by which a T-dependent vaccine response can be induced. The polysaccharide-specific B cells internalize the conjugate and act as their own antigen-presenting cells by displaying peptides of the protein conjugate on their own surface MHC class II molecules. T cells specific for the conjugate provide help for the B cells to enable isotype switching and formation of memory B cells. In contrast to polysaccharides, protein antigens typically activate B cells in the lymphoid follicles of the spleen or lymph nodes.[68]

1.4.6 Immunosenescence

Bacterial and viral infections, such as urinary tract infections, pneumonia, sepsis, influenza and herpes zoster are both more common and more severe among the elderly (> 65 years of age).[72] This has many reasons, including age-associated physiological and anatomical changes, co-morbidities, malnutrition, but also alterations of the immune system with a decreased capacity to eliminate infections. The latter phenomenon is referred to as immunosenescence.

One theory behind the immunosenescence is that chronic infections, typically with persistent viruses such as cytomegalovirus (CMV), cause an exhaustion of the immune system by recurring reactivations throughout life and a gradual decrement in the T cell repertoire.[73] In parallel, elevated levels of pro-inflammatory molecules such as interleukin 6 (IL-6), tumor necrosis factor α (TNFα) and CRP have been described in elderly populations. This causes a chronic low-grade inflammatory state, "inflamm-aging", which is associated with atherosclerosis and other age-related conditions and may augment the tissue-damaging effect of infectious agents in the elderly.[73-75]

Immunosenescence affects both acquired and innate immunity. Regarding the innate immune system, these changes have been shown to include cells as well as soluble mediators such as cytokines, chemokines and reactive oxygen species. [74] Age-related dysfunctions of neutrophils include reduced chemotaxis, phagocytosis, production of free radicals, and increased susceptibility to apoptosis.[75] Antigen-presenting cells such as dendritic cells and monocytes/macrophages have decreased capacity to ingest and present antigens, resulting in reduced T cell activation and proliferation.[73, 74] Macrophage functions may also be compromised with decreased phagocytic capacity and killing of bacteria through oxidative burst.[76] Increased numbers of NK cells feature in the elderly,[77] possibly as a compensatory mechanism for decreased cytolytic capacity.[74, 76]

A fundamental change in immunosenescence is the involution of the thymus. This causes a decreasing output of naïve T cells and consequently, a restricted diversity of CD4+ and CD8+ T cells with a reduced capacity of the host to respond to new antigens.[74] Instead, there is a shift towards accumulation of antigen-experienced memory and effector T lymphocytes. In the aging individual, these T cells may express lower levels of co-stimulatory molecules such as CD28 and CD40L, and reduced production of the growth factor IL-2, the net result being a reduced capacity of CD4+ T cells to stimulate B cell proliferation and antibody production. The repertoire of CD8+ T cells is often dominated by clones of effector cells, typically CMV-specific.[78] The thymus involution also reduces the output of T regulatory cells, which may contribute to inflammation and autoimmunity.[79]

Similar to the T cell line, naïve B cells decline in number with aging while primed memory cells seem to maintain their levels.[73, 74] Clonal expansions of memory B cells with a decreased susceptibility to apoptosis may limit the diversity of the B cell repertoire, and reduced numbers of plasma cells in elderly have been described.[80] Also, IgM memory B cells decrease with age, which affects the defense against encapsulated bacteria.[71, 73] Although total serum immunoglobulin levels seem to be stable during aging, fewer antigen-specific antibodies are produced while autoantibody titers increase.[77, 81] In parallel, antibodies produced in old age are of lower affinity[74, 81] The interplay with other immune cells needed for B cell activation is impaired not only by T

cell changes (see above) but also as a result of decreased co-stimulatory molecules on B cells such as CD40 and CD27.[74]

Humoral and cellular immunosenescence explains the poor immunogenicity and efficacy of vaccination in the elderly. Studies on immune responses to influenza vaccine have shown a 70-90% efficacy in adults < 65 years of age while the level of protection in the age group of > 65 years was at most 50%.[82, 83] Pneumococcal vaccination with a polysaccharide vaccine is protective against invasive pneumococcal disease in adults,[84] but the effectiveness in an elderly population is debated.[84, 85] In a post-hoc analysis of the Dutch CAPiTA (Community-Acquired Pneumonia Immunization Trial in Adults) study involving > 80.000 non-immunocompromised individuals > 65 years of age [86] the predicted efficacy of a conjugated pneumococcal vaccine in preventing pneumonia and invasive pneumococcal disease was 65% in 65-year old subjects, 40% in 75-year old subjects.[87] Studies of the superiority of any of the vaccine types in the elderly have been inconclusive.[88-90] In parallel, naturally acquired antibodies against pneumococci decrease with age.[88, 91]

1.4.7 Immunodeficiency in B cell disorders

Immunodeficiency in B cell malignancies affects various parts of the immune system. It involves dysfunction and abnormalities of B cells, T cells, dendritic cells, and NK cells as well as changes on a molecular level with increased levels of cytokines and other immunologically active compounds that promote the growth of the malignancy and create an immunosuppressive microenvironment.

Polyclonal hypogammaglobulinemia is the classical marker of immune deficiency in B cell malignancies and is seen in most cases of multiple myeloma.[92, 93] A study of untreated WM patients revealed low levels of IgA, IgG, or both in 63%, 58%, and 49% of the patients, respectively.[94] The prevalence of hypogammaglobulinemia in MGUS is lower but still at a level of 25-30%.[95, 96]

The hypogammaglobulinemia in myeloma has been shown to reflect a suppression of CD19+ B lymphocytes, which correlates inversely with disease stage.[92, 97] Reduced levels of B cells have also been demonstrated in WM and MGUS.[98, 99] When MGUS progresses into multiple myeloma, clonal plasma cells (CD19-) successively replace normal plasma cells (CD19+). Olteanu *et al.* found that a mean of 27% of total bone marrow plasma cells in MGUS patients were normal compared to only 3% in myeloma patients,[100] highlighting the deteriorated B cell memory due to reduction of healthy long-lived plasma cells in multiple myeloma. When comparing B cells in patients with myeloma, WM, and MGUS Pilarski *et al.* found an enriched proportion of B cells auto-reactive to polyclonal Ig in multiple myeloma, but not in WM or MGUS. In contrast to

myeloma, levels and function of tetanus-specific B cells were normal in WM and in most MGUS patients.[99, 101]

The T cell repertoire in multiple myeloma, WM and MGUS is characterized by a decreased ratio of CD4+/CD8+ cells, which has been related to reduced numbers of CD4+ cells as well as to a rise in CD8+ cells.[98, 99, 102] Also, an imbalance of the CD4+ T helper cells has been shown in myeloma with an increased ratio of Th1/Th2 cells.[102] As in normal immunosenescence, the T cell diversity is reduced and the remaining clones of CD4+ and CD8+ T cells are expanded; however, this is more pronounced in patients with a B cell malignancy[97, 103] Impaired functional T cell responses are common. Maecker *et al.* compared the CD8+ T cell response to influenza A and Epstein-Barr virus in myeloma patients and healthy controls, and found that less than 30% of the myeloma patients but 67% of the controls responded with increased numbers of virus-specific T cells after stimulation.[104] Studies on regulatory T cells (Tregs) in myeloma and MGUS have had inconsistent results, but a majority have shown increased numbers of Tregs in myeloma with a correlation to disease activity.[93]

Suppression of dendritic cells (DCs) has been described in MGUS but much more markedly in multiple myeloma, where a reduced antigen-presenting capacity and ability to induce T cell proliferation has also been shown.[93, 105] NK cells are increased in MGUS and in the early stages of multiple myeloma.[93, 98] They have an important role in lysis of malignant cells; however, this anti-myeloma effect decreases with progression of the disease through the down-regulation of activating receptors on NK cells and of reduced numbers of NK cell receptors on myeloma tumor cells.[93, 97]

1.5 Infections in B cell disorders

Infectious complications are a leading cause of morbidity and mortality in patients with multiple myeloma.[4, 106] Increased susceptibility to infection is also seen in Waldenstrom's macroglobulinemia and MGUS[5, 6] but the incidence of infections is lower than in multiple myeloma.[107, 108] Besides the disease-induced immunodeficiency, age-dependent decline of immune functions as well as co-morbidities and immobility increase the risk of infections in this mainly elderly population of patients.[73, 109] Other factors that contribute to the vulnerability to infection include treatment-induced immuno-suppression, mucosal damage after chemotherapy and stem cell transplantation, hyperglycemia due to high doses of corticosteroids, transfusional iron overload, and in the case of multiple myeloma, renal failure and respiratory depression caused by fractures of thoracic vertebrae and use of opiates.[106]

MULTIPLE MYELOMA

A recent Swedish population-based study of more than 9000 multiple myeloma patients revealed a 7-fold increased risk of contracting any infection compared to matched controls,[110] which is in accordance with older literature.[107, 111] The incidence of a number of bacterial infections was significantly increased, foremost meningitis (HR = 16.6), sepsis (HR = 15.6) and pneumonia (HR = 7.7), but also herpes zoster (HR = 14.8) and influenza (HR = 6.1).[110]

Bacterial infections, typically pneumonia and sepsis, are predominant in multiple myeloma although viral infections appear to be on the rise.[112] Encapsulated bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae* are classical pathogens, and the increased risk of infection is related to the polyclonal hypogammaglobulinemia seen in a majority of myeloma patients.[106] However, the etiologic spectrum has shifted and gram-negative bacteria, typically *Escherichia coli*, are now the most common pathogens alongside with *Staphylococcus aureus* and *S. pneumoniae*. [4, 18, 112-114] Infections caused by encapsulated bacteria are commonly seen early in the disease while gram-negative and *S aureus* infections have an increasing incidence later on during the disease and predominate in patients with renal failure.[4, 18]

The risk of infection in multiple myeloma is closely related to disease activity and treatment. The highest risk is seen within the first months after diagnosis.[4, 110, 115] Augustson *et al.* showed that 45% of early deaths (< 60 days from diagnosis; 10% of all deaths) were attributable to infections.[115] A 4-fold higher risk of infection has been found in relapsed/progressive disease compared to plateau-phase myeloma.[19] A recent study by Teh *et al.* described a bimodal distribution of bacterial and viral infections with a late rise in incidence at around 70 and 52 months, respectively. This coincides with end-stage progressive disease combined with cumulative immunosuppression following multiple treatment lines.[112]

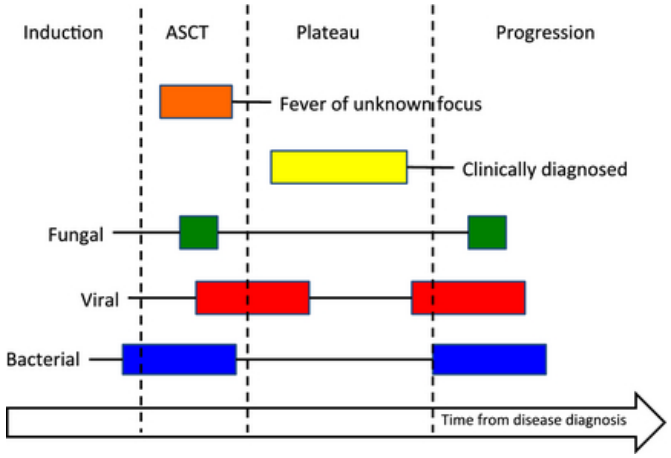

Figure 7. *Risk periods for infection in multiple myeloma. ASCT, autologous stem cell transplantation. Reprinted with permission of Wiley and sons.*[112]

WALDENSTROM'S MACROGLOBULINEMIA

The literature on infectious complications in WM is sparse and consists mainly of case reports of severe infections, foremost bacterial, while systematic reviews are missing. In one retrospective study of clinical features and outcome of 217 patients with a diagnosis of WM, infection was the second most common cause of death next to development of a second malignancy. Of the 52 deaths during the study, 10 (19%) were due to infections, predominantly sepsis and pneumonia.[5] In a study from 1963, Fahey *et al.* compared infections in myeloma and WM patients and found a 3-fold lower incidence in WM patients but similar types of infections.[107]

MGUS

Infections in MGUS have been more studied than in WM. In a recent Swedish population-based study, MGUS patients had a 2-fold risk of developing any infection compared to controls.[6] There was an increased risk of pneumonia, septicemia, osteomyelitis, pyelonephritis, cellulitis, endocarditis, and meningitis, as well as of influenza and herpes zoster with HRs between 1.7 and 3.2. M-protein levels of > 25 g/L were associated with a higher risk of infection but the risk was still elevated in patients with an M-protein of < 5 g/L. Increased incidence of bacteremia in MGUS patients has previously been described in a Danish study with *S. aureus* and *E. coli* as the most common causative agents.[116] Bida *et al.* found an association of MGUS with respiratory bacterial infections, bacterial peritonitis, and mycobacterial infections.[36] Also, there is evidence of an excess mortality due to infections in patients with MGUS.[39, 96] Kristinsson *et al.* showed that patients with MGUS have a 3.4-fold risk of succumbing to a bacterial infection compared to controls.[39]

1.5.1 Infections related to treatment

Treatment-induced infections are a major concern in multiple myeloma. Neutropenia after treatment by classical combinations of chemotherapy and corticosteroids such as melphalan and prednisone typically causes bacterial infections. However, the introduction of more intensive treatment and addition of immunomodulatory drugs has led to prolonged survival but also to increased immunosuppression, and the number and spectrum of infections has widened.[106, 110]

Dexamethasone, especially in high doses, enhances the risk of infections caused by depression of cell-mediated immunity, such as Candida, herpes simplex virus (HSV) and varicella zoster virus (VZV) infections.[93, 106]

Thalidomide and lenalidomide are anti-angiogenic drugs with stimulatory effects on T cells and NK cells.[117] However, these agents may cause higher numbers of severe infections compared to older treatment regimens.[118, 119] Myeloma patients with relapsed disease treated with lenalidomide and high-dose steroids had higher rates of neutropenia and a doubled risk of severe infection compared to patients on dexamethasone alone.[119] Bortezomib has suppressive effects on T cells and is associated with increased risk of HSV and VZV/herpes zoster infections.[106, 120]

High-dose chemotherapy before ASCT is associated with severe neutropenia and a high risk of bacterial infections.[106] Microbiologically confirmed infections afflict about 30% of newly transplanted myeloma patients.[121, 122] The transplant procedure causes prolonged suppression of cell-mediated immunity, and opportunistic infections with CMV, HSV, VZV, *Pneumocystis jiroveci,* Candida, and moulds are seen.[92, 106, 123] Also, respiratory virus (RV) infections such as influenza and respiratory syncytial virus have a high incidence in stem cell transplanted patients as well as in patients on immunomodulatory treatment and are associated with prolonged hospital stay and mortality.[124-127] Lymphocytopenia is a commonly referred risk factor. Teh *et al.* found the highest incidence of RV infections in patients with progressive disease and in recipients of multiple lines of therapy.[127]

Modern treatment of WM generally includes Rituximab, a monoclonal antibody directed towards CD20, which is expressed by all B cells except for plasma cells. The use of Rituximab as monotherapy in lymphomas and rheumatoid arthritis gives an incidence rate of infectious events of 30%.[128] The infectious etiology includes bacterial, viral, and fungal infections, as well as opportunistic infections such as *P. jiroveci* pneumonia and reactivation of HSV, VZV and CMV.[129] Since other treatment options in WM are similar to those in multiple myeloma, including immunomodulatory drugs and ASCT, the spectrum of infectious complications is similar.

1.5.2 Prevention of infection

VACCINATION

Vaccine responses are generally reduced in patients with B cell malignancies. Vaccination against influenza, *S. pneumoniae*, and *H. influenzae* type b (Hib) is recommended but the benefits are uncertain.[106, 130] In a study by Robertson *et al.,* only 19% of myeloma patients developed protective antibody titers against influenza A of two strains and influenza B.[131] Even poorer response rates were found in a Swedish study of patients with hematological malignancies including myeloma, and no effect of repeated vaccination was seen.[132] Influenza vaccination of household members might be of greater importance for these patients.

Initial studies of pneumococcal polysaccharide vaccines in multiple myeloma and WM patients during the 1980s showed suboptimal responses.[133-135] This was later confirmed in a study of 42 myeloma patients who received the 23-valent pneumococcal polysaccharide vaccine (PPV), of whom 38% failed to produce any IgG response.[19] Also, low anti-pneumococcal titers predicted a higher risk of severe infection. Robertson *et al.* found low antibody titers in 61% of myeloma patients vaccinated with PPV, but a good serological response defined as a 4-fold antibody titer increase in 56% of the patients.[131] The 7-valent conjugated pneumococcal vaccine (PCV7) has been evaluated in combination with PPV after ASCT in a small study of mainly multiple myeloma patients.[136] A response rate of 33% was seen after 2 doses of PCV7, and of 78% against the PCV7 serotypes after the PPV booster.

Current American guidelines for pneumococcal vaccination of immunocompromised patients including those with B cell malignancies recommend one dose of the 13-valent conjugated vaccine (PCV13) followed by one dose of PPV at least 2 months later.[137] The European guidelines for ASCT patients recommend three doses of PCV13 followed by one dose of PPV; however, this recommendation is based on studies of allogeneic stem cell transplant recipients.[132] Possible advantages and shortcomings of PCVs vs. PPV in immunocompromised patients are discussed in Chapter 1.7.3.

In contrast to influenza and pneumococcal immunity, levels of Hib antibodies in myeloma patients have been found to be comparable to the general population, before as well as after vaccination, and the benefit of the vaccine has been questioned.[131] Also, after the introduction of the Hib vaccine in childhood immunization programs the incidence of *H. influenzae* type b infections has decreased in all age groups and Hib has largely been replaced by other *Haemophilus* species.[138]

The live vaccine against herpes zoster reactivation is contraindicated in immuno-compromised patients including patients with B cell malignancies, but studies on

recombinant varicella vaccines in ASCT recipients are ongoing and might be a future alternative.[139]

The timing of vaccination with regard to disease stage, treatment and transplantation is an important but largely unexplored research field. In a pilot study by Noonan *et al.* lenalidomide treatment of myeloma patients seemed to augment pneumococcal vaccine responses, indicating that IMiDs might work as vaccine adjuvants.[140] After ASCT, a longer interval between transplantation and vaccination is generally associated with better vaccine responses,[93] but this has to be balanced against the heightened risk of infections during this period of severe immunosuppression. Also, it has been shown that vaccinations against pneumococci and influenza given pre-transplant with a booster dose after transplantation have resulted in better responses.[141, 142] Immunization has not been evaluated in MGUS patients.

ANTIBIOTIC PROPHYLAXIS

Due to the substantial risk of infections in patients with B cell malignancies, antimicrobial prophylaxis is recommended in several situations. Patients treated with high-dose steroids may be given prophylaxis against *P. jiroveci*, preferably with trimethoprim-sulfamethoxazole (TMP-SMX).[15, 106, 143] This should also be considered in rituximab-based regimes in WM patients.[29] Aciclovir prophylaxis is recommended for all patients on bortezomib-containing regimes.[15, 29, 144] Antiviral as well as *P. jiroveci* prophylaxis is given routinely for 6 to 12 months after ASCT.

Antibiotic prophylaxis with TMP-SMX during the first months of disease has previously been shown effective[145] and could be considered according to guidelines.[130, 146] However, a recent study by Vesole *et al.* did not show a benefit of either TMP-SMX or ciprofloxacin vs. patients who were not given prophylaxis.[147] Swedish myeloma guidelines recommend antibiotic prophylaxis in patients with recurrent bacterial infections.[15]

INTRAVENOUS IMMUNOGLOBULIN

One small randomized trial has evaluated the use of intravenous immunoglobulin (IVIg) in multiple myeloma and showed a significantly reduced risk of infections in the treatment arm.[148] The patients were at a stable disease stage and did not receive antibiotic prophylaxis. Current guidelines for B cell malignancies and disorders recommend that IVIg should be reserved for patients with hypogammaglobulinemia and severe recurrent infections.[15, 29, 130] Due to its high cost, a serum level of IgG of < 5g/L has been proposed for IVIg treatment.[106] IVIg has also been investigated during and after ASCT but did not reduce infectious complications.[126, 149, 150]

1.5.3 Treatment of infections

Infections in patients with B cell malignancies, especially multiple myeloma, are very common but may be difficult to correctly diagnose. The spectrum of potential pathogens is wide, and manifestations of infection may be masked by severe suppression of cell-mediated immunity as well as neutropenia due to disease progression and treatment.[106] Also, antimicrobial prophylaxis may complicate the diagnosis of infections. Patients with fever should be treated promptly with broad-spectrum antibiotics covering the most common pathogens (*E. coli, S. aureus, S. pneumoniae*). Aminoglycosides should be used with care in myeloma patients due to renal impairment.[130] In patients treated with high-dose steroids or bortezomib, the risk of viral infections such as HSV and VZV and of fungal infections should be taken into account.

1.6 Pneumococci and pneumococcal infections

Streptococcus pneumoniae is a gram-positive coccoid bacterium of facultative anaerobic nature. The most significant character of almost all pneumococci is the polysaccharide capsule that surrounds the bacterial cell wall and provides protection against the host defense mechanisms of opsonization and phagocytosis. The pneumococci are divided into serogroups and serotypes based on the immunological profile of the capsule. Currently, 97 serotypes have been described.[151] The distribution of pneumococcal serotypes displays natural fluctuations and geographical differences.[152]

Different serotypes have different invasive potential. Serotypes associated with invasive disease are for example 1 and 7F, while serotypes such as 6B and 23F are commonly found in carriage.[153] The rank order of serotype prevalence in invasive disease also varies with exposure and vaccination and forms the basis for selecting serotypes for inclusion in the pneumococcal vaccines.

The polysaccharide capsule is the most important virulence factor of pneumococci. Besides inhibiting complement and immunoglobulin binding to the bacterium, it prevents mechanical clearance by mucus secretion, restricts autolysis, and reduces exposure to antibiotics.[47] Other pneumococcal virulence factors include the pneumococcal surface proteins A and C (PspA, PspC), which act partly by reducing complement activation.[154] LytA, autolysin, is involved in cell wall degradation during bacterial division and lysis, and releases cytotoxins from the cell cytoplasm. The most important cytotoxin, pneumolysin, is a pore-forming exotoxin expressed by most invasive pneumococcal strains and capable of lysing host cells. Pneumolysin has many other functions including induction of ciliostatis and activation of inflammation, which promote bacterial invasion of the host.[47] In addition, pneumococci have a high potential of incorporating genetic material such as antibiotic resistance genes and genes coding for capsular polysaccharides from other pneumococcal strains and streptococcal species, which may help them escape attempts of treatment and prevention.[154]

Pneumococci are the major cause of community-acquired pneumonia world-wide, and bacteremia is seen in up to 15% of adult cases.[151, 155] Carriage rates in adults are around 10%, in toddlers and children in daycare up to 60%.[47] Pneumococcal disease causes close to 1 million deaths per year in children less than 5 years old globally, [155] and the case-fatality rate in invasive disease remains high, especially in the elderly population.[156] The age distribution of infection is characteristic with the highest incidences in infants and old adults. Other risk groups include patients with immune deficiencies, such as primary and secondary hypogammaglobulinemia, complement deficiency, HIV, and functional or acquired asplenia. The marginal zone of the spleen is the most important site for effective killing of pneumococci, and asplenic patients are at significant risk of fulminant invasive infection.[70]

Hematological malignancies typically associated with a high risk of pneumococcal infection are multiple myeloma, chronic lymphatic leukemia, and lymphoma. They encompass a wide spectrum of immune disturbances, but defective antibody production is considered the most important cause for susceptibility to pneumococcal infection. An incidence of invasive pneumococcal disease (IPD) in patients with hematological malignancies of 500-700 cases/100.000 per year has previously been described.[45, 157] However, a recent Swedish study showed an attack rate in multiple myeloma as high as 2238 cases/100.000 per year, compared to 15 cases/100.000 per year in the general population and 45/100.000 in adults ≥ 65 years of age.[156]

1.7 Pneumococcal vaccination

1.7.1 Evaluation of vaccine responses

The benefit of vaccination at the group level is preferably evaluated by clinical endpoints, *i.e.*, reduced incidence of the infectious disease in question. However, such studies are difficult to perform not least in risk groups such as patients with specific immunodeficiencies since they require very large numbers of participants. Instead, for the assessment of pneumococcal vaccine responses, two conceptually different laboratory methods are being used. Quantitative antibody titers are measured by enzyme-linked immunosorbent assays (ELISA) while qualitative opsonophagocytosis assays (OPA) estimate the level of antibody-mediated killing of pneumococci.

The serotype-specific pneumococcal ELISA has been widely used in vaccine studies due to its relative simplicity and cost-effectiveness. The first generation of this assay suffered from low specificity because it also measured antibodies directed against the pneumococcal cell wall polysaccharide (CPS) prevalent in all pneumococci. This was largely overcome by preadsorption of sera with purified CPS, and the specificity of the assay has been further enhanced by the addition of preadsorption of serotype 22F capsular polysaccharide.[158] OPA methods can be of "killing type", estimating the sum of opsonophagocytosis and bactericidal capacity, or flow cytometric, estimating only opsonophagocytic capacity; the killing type is preferred.[159] These analyses are more complex and time-consuming than the ELISA, but represent an *in vitro* model of what are considered the principal host defense mechanisms against pneumococci. Multiplex OPA analyses have been developed and have largely replaced the ELISA in the licensing of new pneumococcal conjugate vaccines.

A pneumococcal antibody concentration of ≥ 0.35 μg/ml, aggregated across all serotypes included in PCV7 has been accepted as a correlate of protection against IPD in infants.[160] This titer has been proposed to correspond to an OPA titer of 1:8 in young

children.[159] Since there are differences between serotypes regarding the amount of antibody required for protection the aggregate correlates have recently been refined, and serotype-specific thresholds for both ELISA and OPA titers in infants have been proposed.[161] Correlates of protection have not been defined for adult populations. Many older adults have high levels of naturally acquired pneumococcal antibodies, and it is anticipated that OPA provides a better correlate of protection than ELISA in this population.[71] In general, it is likely that elderly individuals require higher antibody and OPA titers for protection than what is indicated by the infant thresholds.

1.7.2 Pneumococcal polysaccharide vaccines

Polysaccharide vaccines to pneumococci have been available since the 1970s. The current 23-valent vaccine (PPV) was licensed in 1983. It includes purified polysaccharides from 23 of the most prevalent serotypes in invasive pneumococcal disease (IPD) at the time of licensure. A serotype coverage of 85-95% in IPD has been described in different countries before the introduction of pneumococcal vaccination in childhood immunization programs.[71, 162] The vaccine has been widely used in elderly and immunocompromised patients, and is recommended by the CDC and the WHO, as well as by Swedish authorities.[163-165] The most recent Cochrane meta-analysis from 2013 estimated that PPV has a protective efficacy of 74% against invasive pneumococcal disease in adults.[84] However, the vaccine efficacy against pneumococcal and all-cause pneumonia and mortality in pneumococcal infections is uncertain, especially in elderly patients and adults with chronic diseases, *i.e.*, the populations for whom vaccination is recommended.[71, 84, 85] Due to its T cell-independent mode of action, PPV is poorly immunogenic in children < 2 years of age.

The PPV does not induce an immunological memory; however, pneumococcal antibody titers have been shown to persist above vaccine-naïve levels for at least 5 years and against most serotypes in adults ≥ 65 years.[166]

A subject of much debate in the use of PPV is the risk of hyporesponsiveness, referring to lower immune responses after repeat vaccine doses. This phenomenon was initially described for polysaccharide meningococcal vaccines, and the theory has been that the already existing pool of switched memory B cells is successively depleted after exposure to large amounts of polysaccharide antigen which drives the cells into terminal differentiation without being able to induce new memory cells. An immunologic explanation was launched by Clutterbuck *et al.*, who found decreased levels of memory B cells and higher numbers of plasma cells after PPV vaccination compared to vaccination with a conjugate pneumococcal vaccine.[68] However, primary vaccination with PPV as well as boosting with the same vaccine, has been shown to evoke significant titer increases,[166] and with a longer interval between the doses (5-10 years) immune responsiveness is not depressed.[167] Importantly, the clinical relevance of

hyporesponsiveness is not known. Nevertheless, since the same phenomenon has been demonstrated when PPV has been administered before a pneumococcal conjugate vaccine (PCV), it is recommended that when the vaccines are given in series the PCV should always precede the PPV.[164]

1.7.3 Pneumococcal conjugate vaccines

Pneumococcal conjugate vaccines were developed in order to protect small children against severe disease. The first, seven-valent conjugated vaccine (PCV7) included serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F individually conjugated to the diphtheria carrier protein CRM_{197}. These serotypes caused 80-90% of IPD in children in the United States at the vaccine introduction.[71]

The vaccine was included in the US childhood vaccination program in 2000 and resulted in a dramatic decline in infant IPD.[168] Effects on pneumonia, otitis media and nasopharyngeal carriage in children were also shown.[71] In addition, indirect herd immunity was seen in unvaccinated adults, with a marked decline of IPD in all age groups, not least among persons ≥ 65 years of age, in whom vaccine-serotype and all-serotype IPD had decreased by 92% and 37%, respectively, nine years after vaccine introduction.[169] These effects have continued after the introduction of an expanded conjugated vaccine (PCV13) including 6 additional serotypes (1, 3, 5, 6A, 7F, 19A), see Figure 8.[170]

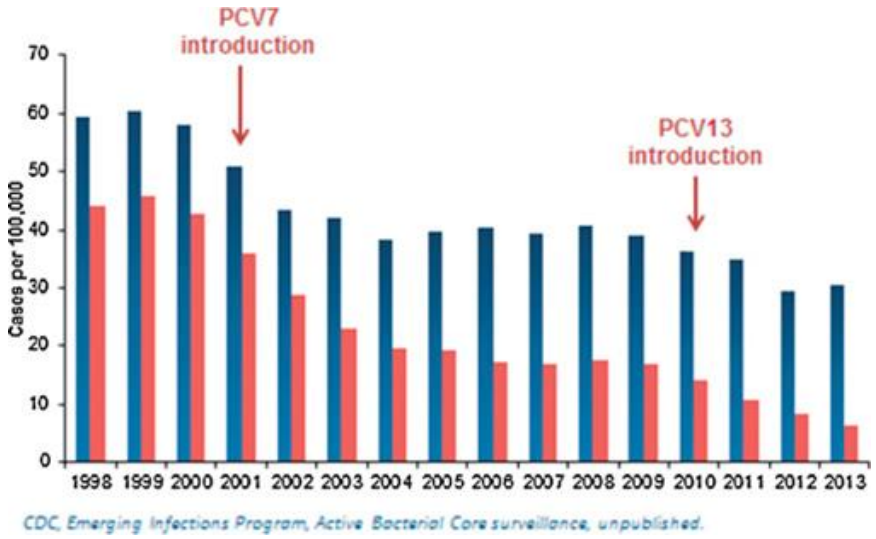

Figure 8. *Changes in the incidence of invasive pneumococcal disease among American adults ≥ 65 years of age, 1998-2013. Blue bars = IPD caused by all serotypes; red bars = IPD caused by PCV13 serotypes. Reprinted with permission of Elsevier.*[170]

PCV7 was incorporated in the Swedish childhood vaccination program between 2007 and 2009 and has later been replaced by 10-valent PCV or PCV13. Due to a partly different serotype distribution, the PCV7 coverage in IPD was only about 50-60% in Swedish children before general childhood vaccination.[162, 171] Despite this, effects similar to those seen in the US regarding infant and herd immunity have recently been described.[151] However, this study could not document a reduction in the incidence of overall IPD in adults ≥ 65 years of age, which was due to a large increase in non-PCV13 strains. This clearly illustrates the matter of serotype shift, which has been a major concern since the introduction of childhood PCV vaccination. Strains that increased after PCV7 introduction, typified by the penicillin non-susceptible 19A serotype, have again decreased after the introduction of PCVs of higher valencies.[151, 170] The Swedish study by Galanis *et al.* describes increasing serotype diversity after PCV13 introduction with a high incidence of strains included only in PPV.[151]

The immunologic advantages of PCVs including the capability of inducing immunologic memory have driven the question of PCV vaccination in adults and elderly. A large clinical trial (CAPiTA) evaluated the impact of PCV13 vaccination on pneumococcal disease in adults ≥ 65 years of age, and demonstrated a vaccine efficacy of 46% and 75% against vaccine-type pneumonia and IPD, respectively.[86] Based on these results, the CDC recommendations on pneumococcal vaccination in elderly were revised, and now include one dose of PCV13 followed by one dose of PPV after 6-12 months.[164] However, it should be pointed out that the CAPiTA study did not include a PPV study arm but only compared PCV13 to placebo. Also, the herd effects of childhood PCV vaccination as well as the shift in serotype distribution, illustrated by the fact that only 32% of IPD cases in an elderly Swedish population were caused by PCV13 strains already 5 years after PCV13 introduction,[151] raise questions as to the future benefit of PCV13 vaccination in adults, and underline the importance of surveillance.

The use of pneumococcal conjugated vaccines in immunocompromised patients is attractive, not least since PPV responses in these patients are often poor, and the conjugated vaccine type has been recommended by the CDC since 2012.[137] However, the literature on PCV superiority to PPV is inconsistent.[172] The CAPiTA study was not designed to show vaccine efficacy in immunocompromised hosts and the number of immunosuppressed patients was small. This aside, no effect on pneumococcal disease of a single dose of PCV13 was shown.[86]

Regarding patients with hematological malignancies, studies of allogeneic stem cell transplanted patients using repeat doses of PCV7 have shown slightly better antibody responses than what has been described after PPV vaccination.[173-176] However, comparisons are difficult since different vaccination regimes and definitions of response have been used. Also, lower serotype coverages of PCV13 and PPV have been described in hematological patients than in the general adult population,[177] and the current

knowledge raises more questions than it gives answers as to how these patients are best protected against pneumococcal infection. The development of conjugated vaccines is technically difficult, and also with a serotype expansion the issue of serotype shift will persist. New vaccine candidates including protein-based antigens and whole-cell vaccines are currently being investigated.[178] However, at present, combination strategies of PCVs and PPV should be the preferred alternative in B cell malignancies and other immunosuppressive disorders in order to obtain the immunologic advantages of PCVs and the broad serotype coverage induced by PPV.

2 Hypotheses and aims

The main purposes of this thesis were to identify risk pathogens in patients with B cell malignancies and disorders, and to investigate their responsiveness to pneumococcal vaccination. An increased knowledge in this field could help in preventing and treating infectious complications, and hopefully contribute to a reduced morbidity and mortality among these immunocompromised patients.

The thesis is based on the following hypotheses:

- Antimicrobial humoral immunity is depressed in elderly patients with multiple myeloma but also in patients with Waldenstrom's macroglobulinemia and MGUS
- Levels of antibodies towards encapsulated bacteria, such as pneumococci are particularly low among these patients
- Pneumococcal vaccine responses are reduced in elderly patients with B cell malignancies and disorders compared to healthy age-matched controls
- A conjugated pneumococcal vaccine evokes higher post-vaccination antibody titers in these patients compared to a polysaccharide pneumococcal vaccine
- ELISA measurements may overestimate post-vaccination anti-pneumococcal immunity in patients with B cell malignancies
- Anti-pneumococcal IgM antibodies may show a better correlation with opsonophagocytic killing activity than IgG antibodies do in patients with B cell malignancies
- Multiple myeloma patients with high disease activity are more susceptible to respiratory viral infections and secondary bacterial complications than patients with low disease activity
- Multiple myeloma patients with positive respiratory virus tests have higher rates of hospitalization and mortality than patients with negative respiratory virus tests

To test these hypotheses, the thesis had the following specific aims:

- Investigation of the serologic immune status to a wide range of bacteria, viruses, and fungi in patients $\geq$ 60 years of age with multiple myeloma, Waldenstrom's macroglobulinemia, and MGUS in comparison to an age-matched control group
- Vaccination of these patients and age-matched controls with one dose of either conjugated or polysaccharide pneumococcal vaccine

- Evaluation of pneumococcal vaccine responses by measurement of serotype-specific IgG and IgM antibodies and of functional antibody activity by opsonophagocytosis (OPA)
- Correlation of anti-pneumococcal IgG and IgM concentrations measured by ELISA, and OPA titers for different pneumococcal serotypes in patients and controls
- Retrospective assessment of the prevalence of respiratory viruses in multiple myeloma patients, and
- Identification of a possible association between positive respiratory virus tests and disease activity, hospitalization, mortality, and other parameters such as age and subsequent bacteremia in these patients

3 Patients and methods

3.1 Patient populations

All study persons in papers I-III were recruited from the Department of Hematology, Uddevalla hospital, Uddevalla, Sweden between May 2008 and March 2009. WHO criteria were used to establish the hematologic diagnoses (multiple myeloma, Waldenstrom's macroglobulinemia, MGUS).[179] In total, 81 persons aged ≥ 60 years were primarily included, 25 with a diagnosis of multiple myeloma (MM), 16 WM, 18 MGUS, and 22 age-matched controls without hematological disorders and from the same geographic area, most commonly the spouse of the patient. Since the focus of these studies was elderly patients with as little immunosuppression as possible, patients < 60 years of age and patients who had undergone high-dose chemotherapy followed by ASCT were excluded. Further exclusion criteria were pneumococcal vaccination or infection (pneumonia, septicemia, meningitis) within a year prior to the study, and previous severe adverse reactions to any kind of vaccination. All participants gave written informed consent.

Two control persons were excluded from the study, one who withdrew, one who had not turned 60 on the day of inclusion. For paper II, three more participants were excluded; one WM patient who dropped out before the second study visit, one MM and one MGUS patient each who turned out to have had pneumonia within a year before inclusion. For paper III, 15 patients from each study group were randomly selected for re-analysis of their post-vaccination serum samples.

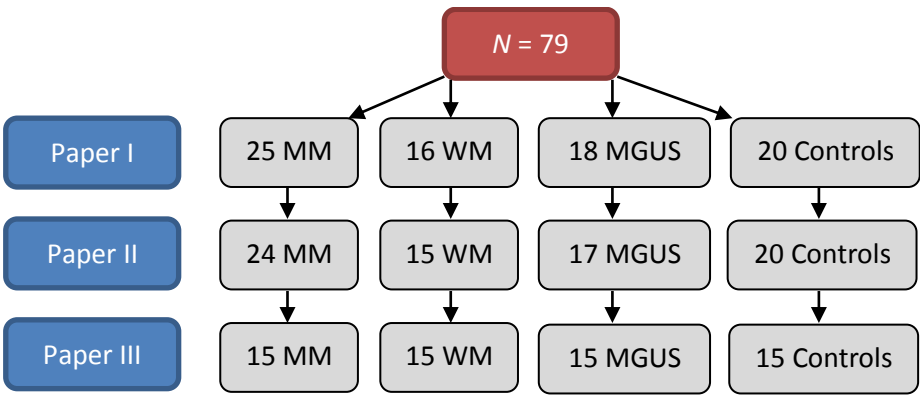

Figure 9. *Patient populations paper I-III.*

In paper IV, all patients with a diagnosis of multiple myeloma who had been sampled for any analysis of respiratory viruses at the Sahlgrenska University Hospital, Göteborg, Sweden between January 2005 and December 2012 were included in retrospect. The cohort comprised 55 patients. Informed consent was waived since we had no contact with the study persons.

3.2 Sampling and vaccination (paper I-III)

The study persons were sampled pre-vaccination and randomized to receive a single dose of either the conjugated pneumococcal vaccine (Prevenar; Pfizer) or the polysaccharide pneumococcal vaccine (Pneumo23; Sanofi Pasteur) given intramuscularly. At the follow-up visit 4-8 weeks later, a post-vaccination serum sample for pneumococcal serology was taken.

Pre-vaccination blood samples were directly analyzed for routine hematological parameters (blood hemoglobin concentration, white blood cell counts, platelet counts, blood differential counts) and serum concentrations of immunoglobulins and M-protein at the local hospital laboratory. Serum was separated and stored at -20^0 C until further analyses.

For paper I, serum was analyzed for antibodies towards 24 different pathogens (bacteria, viruses, fungi, and *Toxoplasma gondii*) by validated methods at the Departments of Clinical Bacteriology and Virology, Sahlgrenska University Hospital. For paper II, pre- and post-vaccination serum samples were transported frozen to Statens Serum Institute, Copenhagen, Denmark and analyzed with serotype-specific pneumococcal ELISA. The opsonophagocytosis assay was performed in part at the National Institute for Health and Welfare, Helsinki, Finland and in part by our research group at the Department of Clinical Bacteriology, Sahlgrenska University Hospital. For paper III, post-vaccination sera were shipped frozen to the Institute of Child Health, University College London, UK for re-analysis by multiplex OPA. Since the antibodies that mediate opsonophagocytosis might be of any isotype, the analysis of serotype-specific IgM by ELISA was additionally performed at the Institute of Child Health in London and added to the previous IgG data.

3.3 Clinical data

For paper I-III, some clinical data were obtained via a patient questionnaire, *e.g.*, previous immunizations, ongoing medication, and smoking habits. At the follow-up visit the patients were asked about adverse events after the vaccination. Further information regarding serious adverse events up to three months after the study termination, as well

as information about disease type and previous and ongoing chemotherapy or immunomodulatory therapy was retrieved from the patients' records.

For paper IV all analyses of respiratory viruses were extracted from a larger database containing all clinical virological assay results (n = 9885) registered for patients with a confirmed diagnosis of multiple myeloma between 1990 and January 2013 at the Sahlgrenska University Hospital. A multiplex PCR method for analysis of respiratory viruses was introduced in 2005 at the Department of Clinical Virology. Because of this, we chose to investigate the database for respiratory virus analyses from January 2005 until the last registered sample in September 2012. The study stop date was set three months later.

Patient data regarding age, sex, serum (S)-IgG and M-protein concentration, white blood cell counts (WBC), blood cultures, autologous stem cell transplantation, hospitalization, and death were retrieved from medical records. The recorded blood tests were selected as close to but not more than four weeks from when the respiratory virus sample was collected. Blood cultures taken within a week from the respiratory virus test were registered.

3.4 Laboratory methods

3.4.1 Serotype-specific ELISA (IgG, IgM)

In paper II, serotype-specific antibodies towards the seven pneumococcal serotypes present in the two study vaccines (4, 6B, 9V, 14, 18C, 19F, and 23F) were measured by an in-house ELISA at Statens Serum Institut, Copenhagen.[180] ELISA microplates were coated with 100 µl pneumococcal polysaccharide in phosphate buffered saline (PBS) overnight at 4-6^{0}C and then washed with a washing buffer containing PBS and 0.1% Tween 20. Sera were blocked for the pneumococcal-specific C-polysaccharide (CPS), which occurs naturally in human serum and may interfere with the detection of capsular polysaccharides, by adding 50 µl of a 0.2% CPS solution to 1 ml serum. Serum was tested in two-fold dilutions starting at 1:100.

100 µl serum was added to each microplate well followed by 1.5 h incubation at room temperature. The plates were washed before addition of 100 µl conjugate (horseradish peroxidase-conjugated rabbit anti-human IgG, swine anti-rabbit IgG), again incubated and washed, and finally 100 µl of substrate (*o*-phenylenediamine) was added. After 30 min of incubation on a shaker, the enzyme reaction was stopped by adding H_2SO_4. Optical densities (ODs) were measured at 490 nm. A local reference serum calibrated to the US standard reference serum 89-SF was used for creating a standard curve.

In paper III, sixty post-vaccination sera were assayed at the Institute of Child Health, University College London, for serotype-specific IgM antibodies to serotypes 4, 6B, 14 and 23F using an in-house ELISA, adapted from the WHO reference IgG ELISA.[181] IgM plates were incubated with alkaline phosphatase-conjugated goat anti-human IgM antibody (SIGMA, Saint Louis, MO, USA) at 1:3000 in antibody buffer for 2 hours at room temperature. Antibodies were detected following incubation with substrate solution and ODs were read at 405 nm.

3.4.2 Opsonophagocytosis

In paper II functional antibody activity was investigated by a modified multiplexed opsonophagocytosis assay (OPA) as previously described.[182, 183] The first 16 sera were analyzed for four pneumococcal serotypes (4, 6B, 14, 23F), the remaining samples with the same method but only for serotypes 4 and 14. Pneumococcal strains of serotypes 4, 6B, 14, and 23F made resistant to one of four antibiotics (optochin, spectinomycin, streptomycin, trimethoprim) were obtained from BEI Resources (Manassas, VA, USA), promyelocytic HL-60 cells used as phagocytes were obtained from ATCC (Manassas, VA, USA), and baby rabbit complement from Invitrogen (Lidingö, Sweden). The HL-60 cells were cultivated for about 8 weeks and investigated for viability and by flow cytometry for surface receptors CD11b, CD35, and CD71 weekly to decide when they fulfilled the acceptance criteria for differentiation.

For the OPA, heat-inactivated serum samples (20 µl) were tested in eight 3-fold dilutions in 96-well plates. A 10 µl-suspension of target pneumococci was added to each well after centrifugation and washing twice with opsonobuffer (Hanks' balanced salt solution (HBSS), sterile water, and fetal bovine serum), and dilution to the proper bacterial density (2 x 10^5 CFU/ml). Meanwhile, HL-60 cells were washed with HBSS twice. After incubation of the serum samples with bacteria for 30 min at room temperature, 10 µl of complement and 40 µl of HL-60 cells (8 x 10^5 cells) were added to each well followed by incubation at 37^0 C in 5% CO_2 with shaking 220 rpm for 45 min. The microtiter plates were then placed on ice and 10 µl of the final mixture from eight wells was plated on four different antibiotic-containing agar plates (Todd-Hewitt broth-yeast extract, THYE). The plates were incubated overnight at 37^0 C in 5% CO_2, photographed, and the number of bacterial colonies counted manually. Opsonization titers were defined as the inverse of the serum dilution that killed 50% of the bacteria. The lowest OPA titer that could be determined was 4.

Each plate contained four complement controls without serum and two bacterial controls without serum and complement. Six patients were treated with antibiotics during the study. To exclude the possibility that residual concentrations of antibiotics had interfered with the OPA the bactericidal effect of these sera was tested alone (test wells containing serum, bacteria, and buffer) and compared to the complement controls

of the standard assay (test wells containing bacteria, HL-60 cells, complement, and buffer). Since no significant OPA titer differences were found, these samples were not excluded from the study.

The OPA performed in London for paper III used the same multiplexed killing-type method with slight modifications.[184]

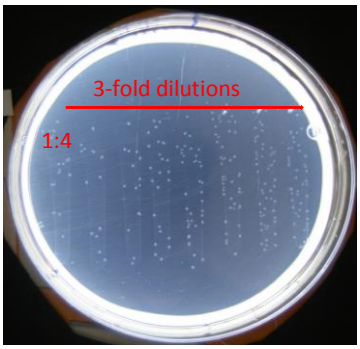

Figure 10. *THYE plate with eight 3-fold dilutions of serum from left to right.*

3.4.3 Multiplex PCR

The multiplex real-time PCR for respiratory viruses used in paper IV is an in-house method developed at the Department of Clinical Virology, Sahlgrenska University Hospital.[185, 186] It was introduced in 2005 and initially analyzed influenza A and B viruses, parainfluenza virus 1-3, metapneumovirus, and respiratory syncytial virus, but has gradually expanded to include rhinovirus, enterovirus, adenovirus, coronavirus (CoV)-229E, CoV-OC43, CoV-NL63, CoV-HKU1, bocavirus, and also *Mycoplasma pneumoniae, Chlamydia pneumoniae,* and *Bordetella pertussis*. Primers and probes were obtained from the literature or developed in the local laboratory.

In the most updated version of the method,[186] nucleic acid was extracted from 200 µl of specimen by a Magnapure robot (Roche Diagnostics, Mannheim, Germany) using the Total Nucleic Acid isolation kit and eluted in a 100 µl volume. A volume of 5 µl was used for the PCR, which was performed in a 7900 real-time (RT) PCR system (Applied Biosystems, Foster City, USA) in 8 parallel reactions of final volumes of 20 µl containing primers and RT-PCR master mix (Applied Biosystems) besides the nucleic acid. Reverse transcription was performed at 46^{0}C for 30 min. After 10 min of denaturation at 95^{0}C, the PCR was performed in two steps (15 s 95^{0}C, 60 s 58^{0}C) and for 45 cycles. As multiplexing tends to decrease the amplification, the reagents were evaluated in comparison to separate reactions by the use of pUC57 plasmids with inserts of the targeted viral sequences (GenScriptCorp, Piscataway, NJ, USA). The combinations were accepted if the cycle threshold (Ct) value was not more than two cycles higher than in a singleplex reaction.

3.5 Statistical methods

3.5.1 Univariate analyses

Median antibody titers were calculated in paper I, geometric means with 95% confidence intervals for pneumococcal ELISA and OPA titers in papers II and III. Antibody and OPA titers below the detection limit were set to 0.5 times this limit; antibody titers above the detection limit were assigned the titer of this limit for statistical calculations. In papers I-III, the Mann-Whitney U test was used to compare means between groups. Paper IV only included patients with the same diagnosis (multiple myeloma). We assumed a normally distributed cohort and in this case used the Student's t test for comparisons of means. The Wilcoxon signed rank test was used for comparisons of paired data, Fisher's exact test for comparisons of categorical variables, and the Spearman rank correlation test for determination of correlations between datasets. All analyses were performed using Graph Pad Prism software (GraphPad, San Diego, CA, USA). A P value of < 0.05 was considered to be statistically significant except in paper I, where a significance level of $P < 0.01$ was chosen to compensate for multiple analyses.

3.5.2 Multivariate analysis

In paper II a multivariate projection analysis, Orthogonal Partial Least Squares (O-PLS) to latent structures, was used (SIMCA P, Umetrics, Umeå, Sweden). This is a method of visualizing patterns in complex datasets and gives a picture of how the data covariates rather than calculating exact P values. O-PLS is a regression and prediction variant of principal component analysis that relates X data to Y variables and separates the data into predictive and uncorrelated information.[187] We used the vaccine response as measured by OPA titers to pneumococcal serotype 14 as Y variable and all remaining patient data as X variables. X variables that were positively or negatively associated with Y in the multivariate model were further analyzed by univariate methods.

3.5.3 Survival analysis

In paper IV overall survival was calculated using the Kaplan-Meier log rank method for comparison of curves in patients with ≥ 1 positive RV test vs. patients with no positive RV test (Sigma Plot 12.5, Systat Software Inc, San José, CA, USA). Overall survival was calculated from the first and from the last respiratory virus test, respectively. Follow-up time was defined as the date of these tests to the study stop date or to date of death if earlier.

4 Results and discussion

4.1 Humoral immunity to infectious agents in B cell disorders (paper I)

In paper I we investigated the prevalence and levels of serum IgG antibodies towards 24 different pathogens in 79 study persons; 25 with a diagnosis of MM, 16 with WM, 18 with MGUS, and 20 control persons.

The study groups were comparable regarding sex and there were no significant age differences between the groups. However, the median age was somewhat higher in MM and WM patients (77 years) than in MGUS patients (71 years) and control persons (69 years). Since the spectrum of infections among elderly overlap with that of patients with B cell malignancies,[4, 72] and a decline in specific antibody titers with increasing age has been shown,[72] it is possible that these differences affected our results. A large number of the patients had hypogammaglobulinemia, which was expected in the MM (92%) and WM patients (44%), but higher in the MGUS patients (56%) than what is commonly described (25-30%).[95] Sixteen MM patients (64%) and one WM patient had ongoing chemotherapy or immunomodulatory therapy, while three MM patients (12%) and eight WM patients (50%) were treatment-naïve.

In general, the MM patients showed the most depressed pathogen-specific antibody titers, which was expected and in accordance with previous findings of infectious susceptibility in B cell disorders.[107, 108] Treatment-induced immunosuppression clearly contributed, since almost two thirds of the MM patients had ongoing therapy. Moreover, there was a tendency towards higher antibody titers among the treatment-naïve MM and WM patients. On the other hand, MM patients are known to have a continuously increased risk of infection including during the plateau phases of the disease.[19] WM and MGUS patients also had significantly suppressed humoral immunity to a number of pathogens when compared to the control subjects, with surprisingly low antibody levels in especially the MGUS group. This certainly mirrors the high degree of hypogammaglobulinemia among our MGUS patients but also supports the notion that these patients, despite a non-malignant underlying B cell condition, are at increased risk of contracting and succumbing to infectious diseases.[39, 116] Co-morbidities such as autoimmune diseases and other types of malignancies, which are seen in increased numbers in MGUS patients, might contribute to the infectious susceptibility.[36]

Four antibody patterns could be discerned from our serological analyses. The first pattern, a stepwise antibody titer increase from MM patients to control subjects, was seen for pneumococci, *S. aureus* alpha-toxin, tetanus toxoid, diphtheria toxoid, VZV, and mumps and rubella viruses.

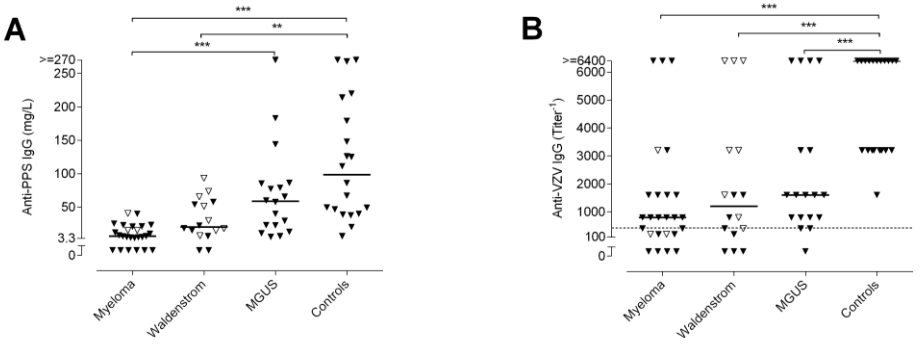

Figure 11. *Antibody titers to pneumococci and VZV. Each triangle represents one individual. Open triangles represent treatment-naïve MM and WM patients. Horizontal bars denote study group medians. (A) Anti-pneumococcal IgG to pneumococcal polysaccharide (PPS). (B) Anti-VZV IgG. The dotted line indicates the cut-off level for protective immunity (400). **, P < 0.01; ***, P < 0.001.*

Regarding pneumococci, serum antibody titers were 15-fold lower among MM patients and 5-fold lower among WM patients compared to the control group ($P < 0.0001$, $P = 0.002$, respectively). Low levels of baseline anti-pneumococcal antibodies have previously been described in MM patients and have been associated with severe pneumococcal disease.[19, 131] WM and MGUS patients have an increased incidence of pneumonia, presumably caused by pneumococci.[5, 6] This fits with our findings of depressed antibody titers in all disease groups and raises the question of pneumococcal vaccination in elderly patients with B cell disorders. Staphylococcal alpha-toxin antibody levels were lower than an age-stratified median in all three patient groups, which might have a clinical implication, since low initial antibody titers have been shown to correlate with complicated bacteremia and high mortality.[188] Protective antibody titers to tetanus and diphtheria were surprisingly low in all study groups highlighting the need for (re)vaccination in this age group at possible exposure to the pathogens. Immunity to tetanus ranged from 8% in MM patients to 65% in control subjects, immunity to diphtheria from 4% in MM patients to 35% among the controls.

VZV antibody titers were 8-fold higher in control subjects than in MM patients ($P = 0.006$). In contrast to the other investigated viruses, there was a loss of humoral immunity in MM as well as in WM patients with a seropositivity of 68% in MM and 75% in WM patients, while MGUS patients and controls reached normal adult levels (94% and 100%, respectively). VZV antibodies have been shown to neutralize the virus

at inoculation sites upon re-exposure.[189] VZV reactivation in the form of shingles is common in the elderly and of increasing incidence in MM and WM patients with the use of bortezomib,[72, 106] but has been attributed to diminished cell-mediated immunity rather than loss of antibodies.[189] However, it is plausible that the patients with the most depressed humoral immunity also had impaired T cell functions, and our results support the use of prophylactic aciclovir in these patients.

The second antibody pattern displayed more depressed titers in MM and MGUS patients than in the WM and control groups, and included *S. aureus* teichoic acid, *Moraxella catarrhalis*, Candida, Aspergillus, and measles virus. Again, the MM group had the most suppressed antibody levels to all antigens.

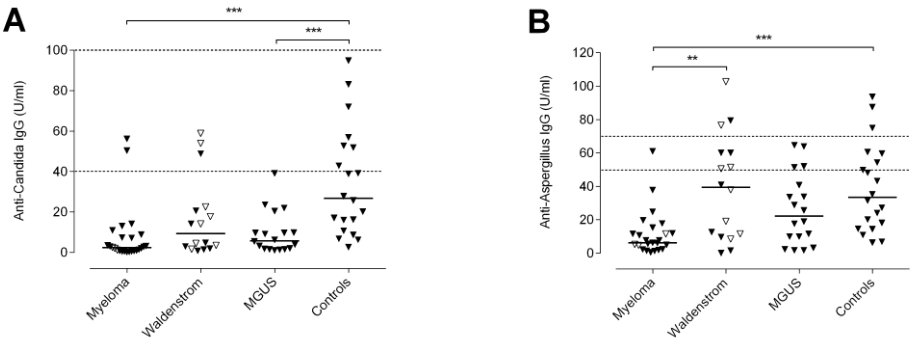

Figure 12. *Antibody titers to Candida and Aspergillus. Each triangle represents one individual. Open triangles represent treatment-naïve MM and WM patients. Horizontal bars denote study group medians. (A) Anti-Candida IgG. (B) Anti-Aspergillus IgG. Titers between the dotted lines are considered borderline, titers below the lower line negative, titers above the higher line positive, in the case of Candida with respect to ongoing infection. **, P < 0.01; ***, P < 0.001.*

Regarding staphylococcal teichoic acid, a 4-fold higher antibody level was seen in the control group compared to the MM group ($P < 0.0001$), and only two MM patients reached the age-stratified population median. The background antibody titers to fungal antigens were very low especially in MM but also in MGUS patients, as shown in Figure 12. Fungal infections typically affect patients with B cell malignancies following prolonged chemotherapy-induced neutropenia, high-dose corticosteroid treatment, and stem cell transplantation.[106] Anti-Candida antibodies have a protective potential regarding disseminated disease,[190] and our results suggest an increased susceptibility among all patient groups.

The third antibody pattern showed measurable and comparable antibody levels among all study groups, and included *H. influenzae* type b (Hib), Borrelia, CMV, Epstein Barr virus (EBV), HSV1, human herpesvirus type 6 (HHV6), and Toxoplasma. In contrast to

pneumococci and staphylococci, Hib antibody concentrations did not differ significantly between the study groups (Figure 13A). Although no participant reported previous vaccination, 80% of MM patients, 88% of WM patients, and 100% of MGUS patients and controls had antibody titers above the generally accepted minimum level for protection against invasive Hib infection (> 0.15 mg/L). This is comparable to a healthy adult population.[191] Hib infections have been frequently reported in older studies of MM patients,[18, 111] which could still motivate vaccination. However, after the introduction of Hib vaccine in childhood immunization programs, Hib has been largely replaced by other, non-capsulated *H. influenzae* strains also in adults.[138]

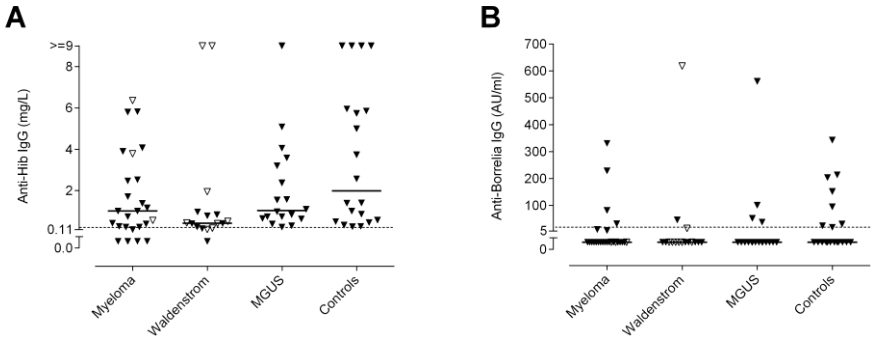

Figure 13. *Antibody titers to H influenzae type b (Hib) and Borrelia. Each triangle represents one individual. Open triangles represent treatment-naïve MM and WM patients. Horizontal bars denote study group medians.(A) Anti-Hib IgG. The dotted line indicates a minimum protective antibody level (0.15). (B) Anti-Borrelia IgG. The dotted line indicates the seropositivity level (15 AU/ml).*

Many of our study persons live in rural areas on the Western coast of Sweden, where Borrelia is endemic and the cause of both clinical and subclinical infections. We found an overall seropositivity rate of 27% with cases in all study groups and unexpectedly high antibody titers (Figure 13B). No patient reported previous invasive borreliosis or ongoing symptoms; a few had been treated previously for erythema migrans. Borrelia antibody titers are known to persist over many years,[192] and our findings illustrate the difficulties of using serological analyses to diagnose current Borrelia infection.

Antibody titers to HSV1, CMV, and EBV were high in all study groups with an overall seroprevalence of 86%, 75%, and 84%, respectively. Viral antibody levels are known to be stable over time,[193] which was confirmed in our study and did not seem to be drastically affected by the immunosuppressed state of the patients. With the exception of VZV, we found high and virtually comparable seroprevalence rates among the study groups also for the other investigated viruses (measles, mumps, rubella, HHV6), although there were group differences regarding the absolute antibody titers. Actually, antibody titers to CMV and HSV1 tended to be higher in the patient groups than among

the controls, which might reflect a higher number of viral reactivations in the immunocompromised hosts resulting in antibody boosting.

The last antibody pattern that was discerned from our data was characterized by an absence of antibodies in all study groups and rather reflected lack of exposure to the pathogens (*i.e.*, HSV2 and various gut pathogens) than waning antibody titers. Antibodies towards the streptococcal antigens streptolysin O and DNase B were seen in a few individuals and of low levels, which is consistent with previous findings in adult populations of developed countries.[194]

In summary, we found suppressed humoral immunity among our disease groups to many of the investigated pathogens, in particular to *S. aureus*, pneumococci, and VZV. Not only MM but also WM and MGUS patients showed clearly depressed specific antibody titers, which underlines an increased susceptibility to infections in these various B cell disorders.

4.2 Pneumococcal vaccination in B cell disorders (paper II)

In paper II we investigated pneumococcal vaccine responses to a single dose of either the 7-valent conjugated vaccine (Prevenar; PCV7) or the 23-valent polysaccharide vaccine (Pneumo23; PPV) as measured by serotype-specific ELISA and opsonophagocytosis (OPA) in elderly patients with MM, WM, and MGUS compared to age-matched controls.

The study participants were the same as in paper I except for one drop-out and two who fulfilled the exclusion criteria, see Patients and methods. Clinical data are shown in Table 1. Almost two thirds of the MM patients (63%), but only one WM patient had ongoing chemotherapy or immuno-modulatory treatment at the time of vaccination. Hypogammaglobulinemia was seen in 92% of MM patients, 40% of WM patients, and 53% of MGUS patients.

Table 1. Study group characteristics.

	Multiple myeloma	Waldenstrom's macro-globulinemia	Monoclonal gammopathy of undetermined significance	Healthy controls	All groups
No. of subjects	24	15	17	20	76
Median age, years (range)	76 (62–88)	75 (62–88)	71 (60–80)	69 (61–83)	74 (60–88)
Female sex, *n*	12	9	11	11	43
Smokers, *n*	1	2	2	0	5
Previous pneumococcal vaccination, *n*	3	3	3	3	12
WBC median, $\times 10^9$ L^{-1} (range)[a]	4.3 (1.3–9.4)	7.1 (2.6–18)	5.9 (3.0–8.8)	6.6 (4.2–11.7)	-
S-IgG median, g/L (range)[b]	1.7 (0.1–8.0)	7.5 (1.0–11)	5.3 (0.3–11)	11 (8.2–19)	-
M-protein median, g/L (range)[c]	27 (0.7–49)	15 (3.0–28)	11 (0.5–28)	0 (0–0)	-
Hypogammaglobulinemia, s-IgG <6.1, *n*	22	6	9	0	37
Ongoing immunomodulatory therapy, *n*	15	1	0	0	16
Treatment regimens, *n*					
Melphalan + prednisone	5	0	0	0	5
Cyclophosphamide + dexamethasone	6	0	0	0	6
Pulse steroids	2	0	0	0	2
Thalidomide	1	0	0	0	1
Bortezomib[d]	2	0	0	0	2
Fludarabin	0	1	0	0	1
Treatment-naïve subjects, *n*	3	8	17	20	48
Ongoing antipyretic therapy[e], *n*	6	3	7	6	22
Given vaccine type, *n*					
PCV7[f]	12	8	8	9	37
PPV	12	7	9	11	39

[a] WBC, white blood cell count; reference level in blood = 3.5–8.8 $\times$ 10^9 L^{-1}.
[b] S-IgG, serum immunoglobulin G; reference level in serum = 6.1–14.9 g/L.
[c] M-protein, monoclonal protein.
[d] One MM patient had a combination regimen of bortezomib, pulse steroids, and melphalan.
[e] Paracetamol or non-steroid anti-inflammatory drugs.
[f] PCV7, 7-valent pneumococcal conjugate vaccine; PPV, 23-valent pneumococcal polysaccharide vaccine.

As expected, with respect to their profound immune suppression, pneumococcal IgG concentrations before as well as after vaccination were the lowest among the MM patients. The same stepwise antibody pattern as was shown by using a multiplex serotype pneumococcal ELISA in paper I was evident at the serotype level, with the lowest antibody titers in MM patients followed by WM, MGUS, and controls. Data for responses to pneumococcal serotype 14 are shown in Figures 14 A and B.

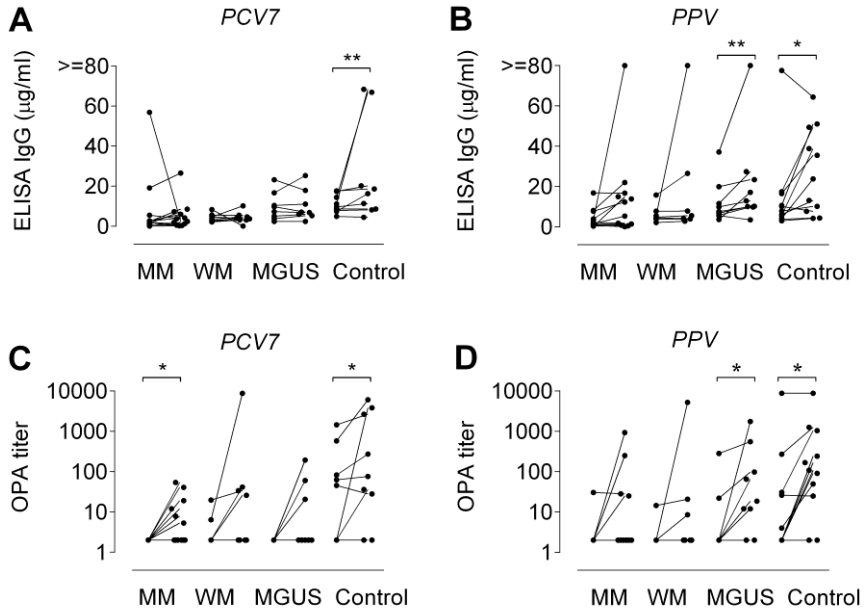

Figure 14. *Anti-pneumococcal IgG and OPA titers to serotype 14 before and after vaccination. MM, multiple myeloma; WM, Waldenstrom's macroglobulinemia; MGUS, monoclonal gammopathy of undetermined significance. (A) and (B), ELISA IgG; (C) and (D), OPA.(A) and (C), PCV7 vaccinees; (B) and (D), PPV vaccinees. Pre- and postvaccination titers for each individual are shown. *, P < 0.05; **, P < 0.01.*

After vaccination, geometric mean IgG concentrations increased modestly in all study groups and irrespective of vaccine type given, with a maximum 4-fold titer increase. MM patients responded with a significant IgG increase to one serotype (18C), WM patients to three serotypes (9V, 18C, 19F), and MGUS patients and control subjects to all four serotypes (9V, 14, 18C, 19F). The poor vaccine responses in MM are consistent with findings in previous studies of the same patient category.[19, 131]

A recurring problem when evaluating pneumococcal vaccine responses by using surrogate markers such as antibody and OPA titers instead of incidence of

pneumococcal infections is the lack of knowledge regarding cut-off levels for immune protection in adults. An IgG threshold of 0.35 μg/ml has been correlated with protection against invasive pneumococcal disease in infants.[160] Adults generally have higher background antibody levels against pneumococci than infants;[195] however, vaccine studies of adults have used IgG cut-offs between 0.15 and 1.0 μg/ml.[88, 173] We chose two levels, 0.5 and 1.0 μg/ml, which are in accordance with previous publications on adult immunocompromised patients.[173, 174, 196] In total, 63% of our MM patients, 73% of the WM patients, 88% of the MGUS patients, and 100% of the control subjects responded with antibody levels ≥ 0.5 μg/ml, and 38% (MM), 60% (WM), 65% (MGUS), and 80% (controls) ≥ 1.0 μg/ml to all seven serotypes after vaccination.

When comparing the vaccine subgroups (PCV7 vs. PPV recipients), baseline as well as post-vaccination antibody titers did not differ significantly for any of the study groups or pneumococcal serotypes, neither did the proportions of subjects with IgG antibody levels ≥ 0.5 μg/ml and ≥ 1.0 μg/ml. Antibody fold titer increases calculated for all seven serotypes combined were low in all study groups (geometric mean fold increases ≤ 2) but there was a significant advantage of PCV7 in the WM group (1.62 vs. 1.32, $P = 0.01$) and of PPV in the MGUS group (1.30 vs. 1.83, $P = 0.03$) while the responses to the two vaccine types were equal in the control group. Regarding the MM group, two patients had very high IgG titers to all serotypes, one before and one after vaccination. These titers could not be confirmed by the functional antibody assay (OPA) and both patients had immunosuppressive treatment as well as hypogammaglobulinemia. The ELISA results were therefore suspected to be falsely high, and when these subjects were excluded, antibody fold increases were significantly higher among the PCV7 recipients also in the MM group (1.79 vs. 1.60, $P = 0.04$).

Opsonophagocytosis assays have been shown to be the best functional correlate of protection against pneumococcal infection.[159] When evaluating an elderly population, the use of an OPA is preferable since the functionality of IgG antibodies has been shown to wane with high age,[197, 198] which also correlates with a higher incidence of pneumococcal disease.[199] We chose to investigate a poorly immunogenic serotype (4), and a highly immunogenic one (14), which were both common in invasive pneumococcal disease in an elderly Swedish population at the time of the study.[162] Our OPA results largely confirmed the results of the ELISA with lower OPA titers in all disease groups compared to the control group before as well as after vaccination, and generally the poorest titers were found among the MM patients, see Figures 14 C and D and Table 2. Significant OPA titer increases after vaccination were seen in MM and MGUS patients for serotype 14, and in control subjects for both serotypes.

Table 2. OPA titers before and after pneumococcal vaccination with either PCV7 or PPV.

Vaccine, study group	Timepoint	Geometric mean OPA titer (95% confidence interval), by serotype		Vaccine, study group	Timepoint	Geometric mean OPA titer (95% confidence interval), by serotype	
		4	14			4	14
PCV7				*PPV*			
MM	T0	2.00 (2.00–2.00)	2.00 (2.00–2.00)	MM	T0	2.00 (2.00–2.00)	2.51 (1.52–4.13)
	T1	5.21 (1.45–18.69)	5.76 (2.58–12.88)*		T1	4.50 (1.52–13.29)	7.68 (1.91–30.82)
WM	T0	2.00 (2.00–2.00)	3.08 (1.51–6.27)	WM	T0	2.00 (2.00–2.00)	2.66 (1.33–5.31)
	T1	4.53 (1.23–16.73)	16.34 (1.45–184.2)		T1	4.30 (1.27–14.55)	10.57 (0.73–152.6)
MGUS	T0	2.00 (2.00–2.00)	2.00 (2.00–2.00)	MGUS	T0	2.00 (2.00–2.00)	4.52 (1.19–17.22)
	T1	11.27 (1.13–112.3)	7.25 (1.51–34.81)		T1	4.81 (1.73–13.39)	34.30 (5.72–205.7)*
Controls	T0	5.74 (1.13–29.2)	24.47 (3.29–181.9)	Controls	T0	3.43 (1.46–8.05)	11.54 (1.83–72.70)
	T1	37.69 (3.60–294.5)*	119.9 (11.4–1260)*		T1	31.33 (5.50–178.6)*	107.5 (19.16–602.6)*

PCV7, 7-valent pneumococcal conjugate vaccine; PPV, 23-valent pneumococcal polysaccharide vaccine.
MM, multiple myeloma; WM, Waldenstrom's macroglobulinemia; MGUS, monoclonal gammopathy of unknown significance.
T0, baseline (*i. e.*, before vaccination); T1, 4–8 weeks after vaccination.
*$P < 0.05$. Comparison of post vs. pre-vaccination antibody concentrations for each study group, respectively; Wilcoxon signed rank test.
P values for comparisons of vaccine subgroups (PCV7 vs. PPV; pre and post vaccination) are all >0.05 (Mann–Whitney *U* test).

An OPA titer of 8 was chosen as cut-off in accordance with previous studies and as a possible equivalent of an IgG level of 0.35 μg/ml. [159, 166, 200] In total, only 8% of the MM patients, 27% of the WM patients, 29% of the MGUS patients, and 55% of the controls reached this level for both investigated serotypes after vaccination. Regarding the vaccine subgroups, there was no trend favoring either of the two vaccines in any study group. The increases in absolute OPA titers were mainly achieved by the PCV7 vaccine in the MM patients, the PPV vaccine in the MGUS patients, and both vaccines in the control group. There were no significant differences in post-vaccination OPA titers, OPA fold titer increases, or proportion of patients with an OPA titer ≥ 8 between the vaccine subgroups.

Since MM, WM, and MGUS are disorders of the B cell line we had anticipated that a conjugated pneumococcal vaccine, which elicits a T cell-dependent response, would be more immunogenic in the investigated patient groups than the polysaccharide vaccine. However, although there was possibly a slight trend towards better responses to PCV7 in the more diseased study groups (MM, WM), our hypothesis could not be confirmed by the study data. The small series of patients in each vaccine subgroup might have contributed to this. Kumar *et al.* reported low but slightly better immune responses in allogeneic stem cell transplanted patients who had received one dose of PCV7 compared to those who had been given PPV.[176] However, the stem cell donor had received one dose of the same vaccine as the recipient before transplantation, which might have enhanced the PCV7 response. The use of a booster dose of either PCV7 or PPV after the first dose of conjugated vaccine would probably have increased the immune responses of our patients as has previously been shown for ASCT recipients and HIV patients.[136, 196] Nevertheless, at the time of this study non-transplanted MM patients and WM patients in our region of Sweden were routinely vaccinated with one dose of PPV, and we wished to evaluate if a single dose of PCV7 was clearly superior to the prevailing vaccination regime, which our results did not support.

The multivariate O-PLS analysis of all data showed an association between good vaccine responses as measured by OPA and high levels of total serum-IgG and WBC, and no previous immunosuppressive treatment. Conversely, hypogammaglobulinemia, high levels of M-protein, and ongoing treatment, in particular with cyclophosphamide and dexamethasone, were negatively associated with vaccine responses. The same tendencies were seen in group-specific univariate correlation analyses and were expected. Hypogammaglobulinemia has previously been related to poor pneumococcal vaccine responses in patients with hematological malignancies.[19, 173] Since 53% of our MGUS patients had hypogammaglobulinemia and the median level of M-protein was high (11 g/L), this is likely to have contributed to the lower vaccine responses in this patient group compared to the control group. Two thirds of the MM patients were on treatment at the time of vaccination, 40% of these with cyclophoshamide and dexamethasone, which partly explains the poor vaccine responses in this study group. In contrast to other studies,[88, 173] we did not find a relation between age or sex and vaccine response.

The vaccines were generally well tolerated among our study participants. In total five unforeseen hospitalizations were recorded during the three month follow-up period after vaccination, none of which was deemed as caused by the vaccine. One MM patient was diagnosed with pneumococcal sepsis two weeks after PCV7 vaccination. The patient lacked a measurable vaccine response, and since the pneumococcal strain was not serotyped it is not known whether it was included in the given vaccine.

The main finding of this study was the suboptimal pneumococcal vaccine responses not only among MM patients but also in WM and in MGUS patients, who have to our knowledge not previously been studied in this respect. We could not demonstrate a superiority of either of the pneumococcal vaccine types given in single dosage. The incongruence between ELISA and OPA results in certain MM patients led us further to the investigations described in paper III.

4.3 Correlation between pneumococcal ELISA and opsonophagocytosis in B cell disorders (paper III)

Paper III describes the correlation between the pneumococcal serotype-specific ELISA and a multiplexed opsonophagocytosis assay in vaccinated elderly patients with MM, WM, and MGUS, and age-matched controls.

Fifteen patients from each disease group and 15 control subjects were randomly selected from the study population in paper II for re-analysis of their post-vaccination sera and correlation statistics. The gender distribution was largely equal between the study groups; 55% of all participants were females. Median age was higher in the MM and WM groups (80 and 75 years, respectively) than in the MGUS and control groups (71 and 68 years; $P < 0.05$), which might have affected our results. Previous studies of pneumococcal vaccination in adults have shown poorer OPA responses in elderly compared to younger adults.[71, 197, 201]

Our OPA analyses confirmed the pattern seen in paper II with lower functional antibody titers among all disease groups than in the control subjects. This was significant for all investigated serotypes (4, 6B, 14, 23F) in WM patients, for three serotypes among the MM patients, and for two serotypes among the MGUS patients. ELISA IgM antibodies were seen at very low levels in the MM patients ($P < 0.05$ for serotype 23F, $P < 0.001$ for the remaining three serotypes vs. control subjects), while the WM, MGUS, and controls had comparable IgM titers. This contrasted to the stepwise pattern again seen for the anti-pneumococcal IgG, with the lowest titers among MM ($P < 0.05$ for all serotypes vs. controls) followed by WM, MGUS, and control subjects.

The given pneumococcal vaccines (PCV7, PPV) were evenly distributed in the study groups. Since no differences in either OPA or ELISA antibody titers were observed between the vaccine subgroups in any of the study groups these data were pooled for the correlation analyses. We found significant correlations between anti-pneumococcal IgG and OPA titers for all four serotypes among the control subjects with correlation coefficients (r) ranging from 0.51 to 0.85. In sharp contrast, the MM patients showed no correlation for any of the serotypes (r = -0.18 – 0.21). WM patients had borderline significance for two serotypes (r = 0.53 and 0.54, $P = 0.06$) while the MGUS patients resembled the control group with significant correlations for three out of four serotypes (r = 0.53 – 0.80). Correlation analyses for serotype 23F are shown in Figure 15.

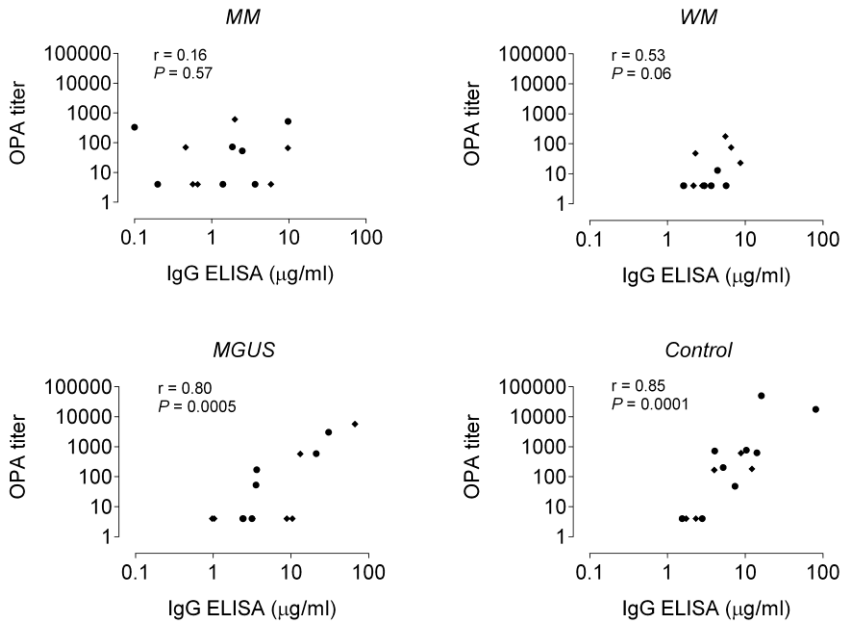

Figure 15. *Correlation between anti-pneumococcal IgG and OPA titers for serotype 23F. MM, multiple myeloma; WM, Waldenstrom's macroglobulinemia; MGUS, monoclonal gammopathy of undetermined significance. Diamonds indicate patients vaccinated with the conjugated pneumococcal vaccine (PCV7), circles indicate patients vaccinated with the polysaccharide vaccine (PPV).*

Anti-pneumococcal IgG levels have been shown to correlate well with opsonic activity in vaccinated children but more poorly in the elderly population.[197, 202] Romero-Steiner *et al.* found weaker correlations in a group of vaccinated elderly adults compared to younger adults, which was explained by lower antibody avidity among the elderly.[197] We found good correlations among our elderly control subjects; however, their median age was lower (68 years) than in the group of elderly adults of the referred study (85 years). Discrepancies in the outcome of pneumococcal ELISA and OPA analyses as in our MM and WM study cohorts have previously been described in other immunocompromised patients such as HIV patients and renal transplant recipients.[203, 204] In contrast, a study of allogeneic stem cell transplanted patients showed significant correlations between anti-pneumococcal IgG and OPA titers in 7/7 investigated serotypes.[205] These patients were considerably younger (mean age, 37 years) than those of our study and had other hematological diagnoses.

In general, elderly adults require higher IgG titers against pneumococci to achieve the same killing capacity as younger adults.[71, 197, 198, 201] Besides a lower antibody avidity, this phenomenon has been ascribed to loss of oligoclonality in response to pneumococcal

antigens, reduced function of phagocytic cells, and decreased levels of anti-pneumococcal IgM as a consequence of deficient IgM memory B cells with increasing age.[71, 206, 207] We found markedly low IgM titers in the MM patients, who had the highest median age among our study groups but who have also been shown to have the most suppressed B cell memory.[100, 101] In contrast, anti-pneumococcal IgM titers in WM patients were relatively high although it was only one serotype that correlated significantly with the OPA analysis. The correlation between anti-pneumococcal IgM and OPA titers was poor and inconsistent in all four study groups, which indicates that lack of IgM was not the main reason for poor OPA function in our study subjects.

We speculate that interference of M-protein with the pneumococcal ELISA resulting in falsely high antibody concentrations could be a reason for the poor correlation between the serological and OPA analyses in our disease groups. This could also explain the high anti-pneumococcal IgM titers in the WM patients, who have an M-protein of IgM subtype. Cross-reactions with M-protein have previously been shown for a variety of laboratory tests including analyses of anti-streptococcal antibodies.[208] Unfortunately, since we had too little serum left to measure the post-vaccination M-protein levels in our patients, we could not test this hypothesis properly. However, it is unlikely to be the only cause for the incongruence between the ELISA and OPA results as our MGUS patients exhibited nearly as good correlations as the control subjects despite relatively high pre-vaccination levels of M-protein. The OPA responses may also have been influenced by disease-related cellular immune defects, which are more profound in MM and WM patients than in MGUS patients.[99]

In summary, this study showed that post-vaccination pneumococcal antibody titers as measured by serotype-specific ELISA correlated poorly with the results of a multiplex killing-type OPA in sera from elderly patients with a diagnosis of multiple myeloma or Waldenstrom's macroglobulinemia.

4.4 Respiratory viruses in multiple myeloma (paper IV)

In paper IV we retrospectively investigated the prevalence of respiratory viruses (RV) in nasopharyngeal samples collected from myeloma patients with respiratory symptoms and examined a possible association with age, sex, disease activity, disease duration, ASCT, sepsis (particularly of pneumococcal origin), hospitalization, and death.

In total, 98 respiratory samples from 55 myeloma patients were identified during the study period. Forty-one of the samples (42%) derived from 24 of the patients (44%) were positive for at least one RV. This is similar to the detection rate of RVs in all unsorted clinical samples that were analyzed with multiplex PCR at the same laboratory between 2006 and 2009,[209] and with previous studies using similar PCR methods and panels of viruses.[210, 211] The most frequently detected virus in our study cohort was rhinovirus, followed by influenza viruses, respiratory syncytial virus (RSV), metapneumovirus, and parainfluenza virus (Table 3).

Table 3. Respiratory tract viruses.

Respiratory tract virus	*n* (%)
Rhinovirus	11 (27)
Rhinovirus +/- enterovirus	2 (5)
Respiratory syncytial virus	7 (17)
Metapneumovirus	5 (12)
Influenza A	3 (7)
Influenza A H1N1	1 (2)
Influenza B	3 (7)
Influenza B + coronavirus	2 (5)
Parainfluenza virus	4 (10)
Adenovirus	2 (5)
Coronavirus	1 (2)

Our findings were comparable to those of two recent studies of other hematologic patients (allogeneic stem cell transplanted patients, hematologic patients with neutropenia). [212, 213] In a majority of studies, influenza viruses and RSV dominate the respiratory viral infectious spectrum,[124, 125, 214, 215] which may in part be explained by the use of older detection methods such as viral culture and antigen detection. PCR methods have been shown to increase the diagnostic yield of respiratory viruses, not least of rhinovirus.[216, 217]

The seasonal distribution of viral isolates in this myeloma cohort showed the same pattern as in the general population of temperate climate zones,[209, 214] with influenza and RS viruses only detected during the winter months while rhinovirus and parainfluenza virus were seen all year round. No epidemics of viral infections were evident in our study as reflected by the relatively few RV analyses that were performed during the study period. Myeloma patients with mild symptoms are normally advised to stay at home and not to seek medical care. It is also probable that a number of the patients with respiratory tract symptoms were not sampled regarding RVs since most of these infections lack a specific treatment.

As myeloma patients have an enhanced risk of contracting bacterial respiratory infections,[106] we expected to find positive blood cultures in relation to RV analyses, both representing secondary infections and differential diagnoses of viral infections. However, although blood cultures were taken in relation to the RV samples in 50% (49/98) of the cases, only two were positive, both for pneumococci, and both preceded by negative RV tests. One reason for the low hit rate might be that most myeloma patients with ongoing treatment including during the post-transplantation period receive antibiotic prophylaxis.

Thirty-six of the 55 patients were sampled once for analyses of RVs, the remaining patients had had between two (n = 7) and eight (n = 1) analyses performed during the study period. Seventeen of the patients were female (31%) and 38 male (69%). Multiple myeloma is slightly more prevalent in men than in women;[14] however, the male predominance is less than what was seen in the RV sampling which could indicate a true gender difference regarding infectious susceptibility or the proneness of the care provider to order these virological analyses.

When comparing patients with and without virus-positive respiratory specimens only two factors displayed a significant difference between the groups: the virus-positive patients were younger (median age 58 years vs. 61 years; P = 0.01) and had shorter disease duration at the time of sampling (653 days vs. 1177 days; P = 0.04). The latter is in accordance with the fact that the incidence of bacterial as well as viral infections in myeloma patients is the highest during the first months after diagnosis.[106, 110]

The incidence of leukopenia (WBC < 3 x 10^9 L^{-1}) was equal in the patients with and without positive RV analyses (24% and 23%, respectively). Lymphopenia and neutropenia have previously been identified as risk factors of severe RV infections.[124, 215] Unfortunately, it was not possible to evaluate the role of specific white blood cells since blood differential counts were missing in many of our cases, but there was a slight trend towards lower WBC in the patients with at least one positive RV sample. In contrast, and in opposition to what we expected, there was a clear tendency towards higher levels of M-protein (median 19 vs. 7.0 g/L; P = 0.08) and lower S-IgG (median 3.3 vs. 4.0 g/L; P = 0.16) in patients with only virus-negative analyses. Possibly, these patients with

more advanced disease were less active and therefore less exposed to community-acquired infectious agents. Additionally, they may have been more liberally sampled for infectious agents as a consequence of their immunosuppression.

Two thirds of the patients had undergone ASCT at the time of viral sampling with a tendency towards higher transplantation rates in patients with positive RV analyses (79% vs. 58%; $P = 0.15$). In a recent study of myeloma patients undergoing ASCT, one third of the patients experienced at least one infectious episode during the first month after transplantation, most commonly a respiratory tract infection with influenza virus and pneumococci as the predominantly identified pathogens.[126]

Fifty-three of the RV samples (54%) were collected in hospitalized patients; twenty (38%) of these were positive with influenza viruses, RSV and rhinovirus being the most common pathogens. Previous studies have reported high frequencies of hospitalization and prolonged hospital stay in patients with hematological malignancies and influenza or RSV infection.[127, 214] We did not find a significant difference in the duration of hospitalization between patients with and without findings of RV in their respiratory secretions. However, since hospitalizations in our study were due not only to infection but also to ASCT and the administration of chemotherapy it is likely that fever and respiratory symptoms had other causes besides infectious complications.

Overall mortality in the study was 47%. Thirteen of the 55 patients (24%) died within three months after a RV analysis, however without a difference between patients with virus-positive and virus-negative samples. Survival analyses could not prove a statistical significance between patients with ≥ 1 RV positive specimen vs. patients with only negative specimens but when looking at 50% survival there was a 400-600 days advantage for the test-negative patients (Figure 16). The trend towards higher mortality in patients positive for respiratory viruses, who also had shorter disease duration, is in accordance with the findings of Blimark *et al.* describing an infection-related mortality of 22% one year after the myeloma diagnosis.[110]

In summary, this retrospective study showed the same detection rate and pattern of respiratory viruses as in the general population. Myeloma patients with at least one virus-positive respiratory sample had lower median age and shorter disease duration than patients with no positive samples. Duration of hospitalization and mortality did not differ significantly between the groups.

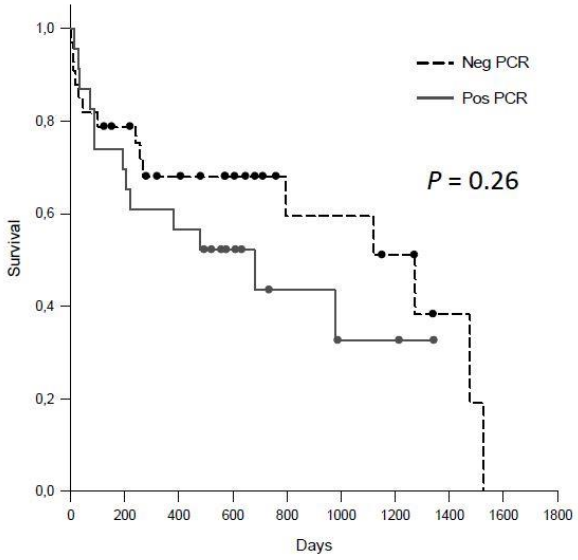

Figure 16. *Overall survival for patients with ≥ 1 positive respiratory virus sample vs. patients with no positive sample. The last positive sample for each patient in the virus-positive group of patients was compared to the last negative test for each patient in the virus-negative group.*

4.5 Methodological considerations

A limitation of the studies described in papers I-III was the relatively few individuals in each study group, especially in paper II where each study group was subdivided into two vaccine groups (PCV7 vs. PPV). However, the studies comprise diagnoses (MGUS, WM) and subgroups of patients (elderly, non stem-cell transplanted) who have previously been sparsely investigated regarding humoral immunity and vaccine responsiveness. Since the prevalence of these B cell disorders is low, especially WM, larger cohorts would probably have required a multi-center strategy, which is desirable for future studies not least in the evaluation of vaccine responses.

In papers I-III there was a discrepancy between the median ages in the study groups such that the MM and WM patients were older than the MGUS patients and the control subjects. The differences were significant in paper III but not in paper I and II. However, this might still have affected the outcome of vaccination as well as the serological and OPA results with poorer responses and titers in the more diseased (and older) study groups.

The clinical data in papers I-III were retrieved from a patient questionnaire and from medical records retrospectively. This is a limitation since the information is dependent on correct documentation and self reports on vaccination status, which are often not reliable.

In paper II, the choice of single-dose vaccination may be considered as controversial regarding the conjugated vaccine since the vaccine effect is boostable and current recommendations advise one dose of PCV13 followed by one dose of PPV in immunocompromised adults.[137] This is the preferred strategy in order to broaden serotype coverage in risk groups. However, as discussed in the results section, at the time of our study the vaccination regime in our region comprised a single dose of PPV for these patients, and we wished to evaluate if a single dose of PCV7 would result in an improved response to the included serotypes. Other limitations of the pneumococcal vaccination study include the absence of a long-term follow-up of the vaccine responses, and the use of an ELISA method which did not employ pre-adsorption of serotype 22F polysaccharide. The latter intervention has been shown to reduce the effect of cross-reacting, non-functional pneumococcal antibodies especially in pre-immunization sera.[218] Further, there was a relatively wide interval between the vaccination and the follow-up sampling (4-8 weeks); however, the number of days showed no association with post-vaccination OPA titers in neither the multivariate nor in the univariate analyses. Paper II comprised multiple statistical analyses and a post-hoc test was considered to reduce the risk of type I error. Since this was not applied in the majority of similar refereed vaccine studies, we chose not to do so either but to use the same statistical methods and significance level (0.05) to be able to compare our results.

A draw-back of paper III is the fact that we were not able to perform analyses of post-vaccination levels of M-protein in the disease groups. This would have been required to test the hypothesis of interference of the M-protein with the ELISA analyses as a reason for the poor correlations between ELISA and OPA results in the MM and WM patients. Another limitation is the use of different laboratories, time-points and methods for the ELISA IgG and IgM analyses. However, both assays were accredited and performed at pneumococcal reference laboratories. The results were not compared with each other but only with the OPA responses.

The limitations of paper IV include the retrospective nature of the study and the relatively low number of respiratory virus samples and patients, which may reflect that clinicians were not prone to order the analyses and/or did not consider respiratory viruses to be of clinical significance in myeloma patients. A larger number of samples would have allowed for more comparisons between the detected viruses, and larger patient groups would probably have resulted in clearer differences between patients with positive RV analyses vs. patients with only virus-negative samples. The differing numbers of respiratory samples collected from the study patients may have skewed the data. Further, the successive expansion of viruses detected by the multiplex PCR method in use during the study period should have influenced the outcome. Finally, we did not investigate the association between virus positivity and immunosuppressive treatment regimens other than ASCT or gather information on symptoms and causes of death, which would be valuable to include as study parameters, especially in a larger patient cohort.

5 Conclusions

Background antibody levels to a large number of microbial pathogens are depressed in patients with B cell malignancies and disorders indicative of an increased infectious susceptibility. This is seen primarily in MM patients but also in patients with WM and MGUS. We identified pneumococci, *S. aureus,* VZV, and fungi (Candida, Aspergillus) as risk pathogens. In contrast, humoral immunity to *H. influenzae* type B and most viral agents was retained in these groups of patients.

Antibody responses to pneumococcal vaccination as measured by serotype-specific ELISA and opsonophagocytosis are suppressed not only in MM patients but also in WM and MGUS. A single dose of conjugated pneumococcal vaccine (PCV7) was not superior to the polysaccharide vaccine (PPV) in our study cohorts. Hypogammaglobulinemia and ongoing chemotherapy were associated with poor vaccine responses in a multivariate model.

Pneumococcal antibody titers as measured by serotype-specific IgG and IgM ELISA correlate poorly with the results of a killing-type opsonophagocytosis assay (OPA) in patients with MM and WM. Our data suggest that ELISA measurements may overestimate anti-pneumococcal immunity in patients with B cell malignancies. The use of an OPA method should be preferred for evaluating pneumococcal vaccine responses in these patients.

Rhinovirus, influenza viruses, and RSV were the most commonly detected respiratory viruses in a retrospective study of MM patients. Patients with virus-positive tests were younger and had shorter disease duration than virus-negative patients. Duration of hospitalization and mortality did not differ significantly between the groups. Although MM patients have an increased risk of RV infections we did not confirm a major impact on morbidity and mortality; however, this needs to be evaluated in larger studies.

6 Future perspectives

Patients with multiple myeloma, Waldenstrom's macroglobulinemia and MGUS are risk groups of contracting and succumbing to infections. Increased knowledge of the spectrum of pathogens, vaccine responses as well as timing of infections and additional risk factors are needed to improve preventive strategies against infections in these patients.

Regarding pneumococcal vaccination of patients with B cell malignancies, the timing of vaccination in relation to disease stage, treatment, and ASCT remains to be elucidated. The finding of augmented pneumococcal vaccine responses in lenalidomide-treated myeloma patients indicates a possible vaccine adjuvant function of this class of drugs,[140] which definitely merits extended investigations. Awaiting future generations of pneumococcal vaccines, the existing conjugate and polysaccharide formulations should be used in series in these patients. Further studies are required to investigate if there would be a benefit of repeat doses of PCVs followed by the polysaccharide vaccine in patients with B cell malignancies. Such studies should include ASCT recipients, since current recommendations on pneumococcal vaccination in these patients are largely based on studies of allogeneic stem cell transplanted patients. Considering the role of immunodeficiency in B cell disorders with respect to vaccine responses, it would be of interest to further investigate B cell subsets, for example IgM memory B cells, which are of major importance in the response to polysaccharide vaccines.[71]

When comparing pneumococcal ELISA and OPA results we found a very poor correlation between the analyses in MM and WM patients. It would be desirable to investigate if our results are reproducible in a larger patient cohort and also in other hematological conditions, for instance chronic lymphatic leukemia. It would also be of interest to test our hypothesis of interference of M-protein with the pneumococcal ELISA analyses, which would require measurement of post-vaccination M-protein levels in parallel with antibody and OPA titers.

In order to better study the prevalence of and morbidity from respiratory viruses in B cell disorders a larger study, preferably prospective, with addition of data regarding causes for hospitalization and death is warranted.

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